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SOME OBSERVATIONS ON CHANGES IN THE BLOOD CONTENT OF THE CAT'S PANCREAS DURING ACTIVITY

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(*Received* 22 *June* 1959)

Claude Bernard (1856) first observed that the pancreas became red and congested during digestion, and it has been generally assumed since then that activity in the pancreas is accompanied by vasodilatation. Experimental evidence is, however, conflicting; and Tankel & Hollander (1957) state in a recent review that 'there is no conclusive evidence that an increase in glandular activity is invariably accompanied by an increase in blood flow'.

The problem is complicated by the work of Bennett & Still (1933), who showed that obstruction to the outflow of pancreatic juice caused an increase in venous outflow from the pancreas: when there was no obstruction, secretion was not accompanied by an increased blood flow. It is therefore important that any method of observing vascular changes in the pancreas should not interfere with the outflow of juice.

We have approached the problem by using a photocell to detect changes in opacity of a portion of the cat's pancreas when transilluminated. This method, which was adapted from that of Holton & Perry (1951) has the advantage of producing very little mechanical disturbance of the pancreas. It must be emphasized, however, that it does not measure blood flow, but is sensitive to changes in the blood content of the tissue under observation. It is probable that blood flow and blood content alter together. But this is not necessarily so: Hilton & Holton (1954) showed that, in the rabbit's ear, capillary vasodilatation can occur with very little increase in blood flow.

In this paper, the words 'vasodilatation' and 'vasoconstriction' mean increase and decrease in blood content of the tissue and do not imply blood-flow changes.

METHODS

Apparatus. The apparatus is illustrated in Fig. 1. A small photocell, *P* (CINTEL V.A. 31), with an end cathode 11 mm in diameter, was mounted in a Perspex plate *B*, attached to a holder *H*. The light source was a 6 W bulb run from an accumulator and mounted on a Perspex rod *R*, which contained a green filter. The lower end of the rod was encased in a polished stainless-steel tube and attached to a Perspex plate *A*. When the pancreas was in position, *A* and *B* were bolted together, with spacers to avoid compression of pancreatic tissue. The outer surface of the Perspex was painted black to prevent reflexion of light on to the photocell from the abdominal contents.

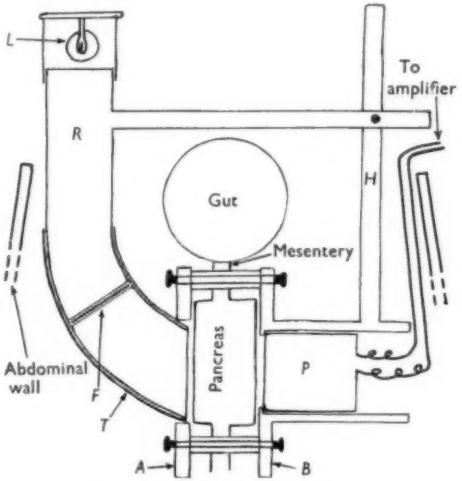

Fig. 1. Diagram of the apparatus. The pancreas was held between Perspex plates *A* and *B* bolted together through holes in the mesentery. Light from the bulb *L* passed down the Perspex rod *R*, via a green filter *F*, through the pancreas to the photocell *P*. The leads from the photocell were insulated with Araldite (Aero Research Ltd.) and were brought out through the abdominal wound. The lower end of *R* was encased in a polished stainless-steel tube *T*. The outside surface of all the Perspex inside the abdomen was painted black.

The method depends upon the fact that an increased amount of blood in the tissue under observation decreases the light reaching the photocell (and vice versa). The loss of light is due partly to scattering and partly to absorption. Since most of the change in absorption is due to change in the amount of haemoglobin, a wave-band of light was selected by using a green filter (Ilford 404, Tricolour Green Micro 3) so that both haemoglobin and oxyhaemoglobin were absorbed strongly and approximately equally. This precaution prevented the results being obscured by possible alterations of the oxygen tension. It was probably unnecessary, however, since similar results were obtained in early experiments in which the green filter was omitted.

The output from the photocell was amplified as described in the appendix and led into one of the two channels of a recording milliammeter (Evershed & Vignoles). The amount of light entering the photocell, and hence the output of the amplifier, with the pancreas at rest, depended upon the particular portion of pancreas used and changes in this light were

only a small proportion of the total. When the pancreas was in position, the initial output of the amplifier, which varied from 3 to 40 V in different experiments, was backed off with a dry battery (see Fig. 7) so that the deflexion of the milliammeter was minimal in the absence of stimulation. Since the sensitivity of the apparatus to a given percentage change of transmitted light varied with the initial setting of the amplifier, the deflexions of the milliammeter were not comparable from one experiment to another. On some occasions the sensitivity was too great, and when this occurred the deflexions of the recorder were decreased by introducing a resistance in series with it. The other channel of the milliammeter was attached to the drop tube and amplifier normally used in conjunction with the Gaddum drop-timer and recorded drops of pancreatic juice.

Preparation of the animal. Cats weighing 2·5–5 kg were anaesthetized with intravenous chloralose after induction of anaesthesia with ethyl chloride and ether. The abdomen was opened, the pylorus tied and the pancreatic duct cannulated. The portion of pancreas chosen for observation lay in the duodenal loop: this presented, in the cat, an area large enough to cover the photocell, and of a suitable thickness for transillumination, but without any visible blood vessels. In addition, this portion of the pancreas was far enough away from the duct to avoid interference with the collection of secretion. Before clamping the pancreas between the Perspex plates, four holes were dissected through the mesentery to accommodate the screws and spacers fixing the plates. The duodenum adjacent to the pancreas under observation was thus supported by the screws holding the Perspex plates together. The edges of the wound were brought closely together; in some experiments the abdomen was filled with liquid paraffin at 38° C. (This kept the preparation in excellent condition, but impeded any subsequent adjustment to the apparatus or pancreas.) The polythene cannula from the pancreatic duct was brought out through the wound and attached to the drop recorder.

The arteries supplying the pancreas arise from the coeliac and superior mesenteric branches of the abdominal aorta. Arterial injections were therefore made into a polythene cannula passed via the femoral artery into the aorta until the tip lay proximal to the origin of the coeliac artery (checked post mortem). Intravenous injections were made into the jugular vein. Blood pressure was recorded with a mercury manometer from the common carotid artery.

Secretin and pancreozymin were obtained from Boots Pure Drug Co., Ltd. Doses are expressed in the units described by Crick, Harper & Raper (1949).

RESULTS

In the absence of stimulation the mean photocell record remained steady, but there were small waves due to changes in the amount of light reflected from the spontaneously contracting intestine. These fluctuations could not be excluded without compressing the pancreas, but did not obscure the observations on changes in blood content.

The effect of injections

Saline (NaCl 0·9 % (w/v)). Rapid arterial injection of saline in a volume of 0·5 ml. or larger decreased the opacity (i.e. simulated vasoconstriction), owing to the replacement of blood with a colourless liquid. This was not shown with smaller volumes (0·3 ml.), nor with intravenous injection. Care was taken to keep the volumes injected arterially not greater than 0·3 ml.

Acetylcholine. Arterial injection of acetylcholine 10 μg produced an evanescent vasodilatation, as is shown in Fig. 2. There was no increase in the volume of pancreatic secretion in response to acetylcholine, even when 100 μg was injected.

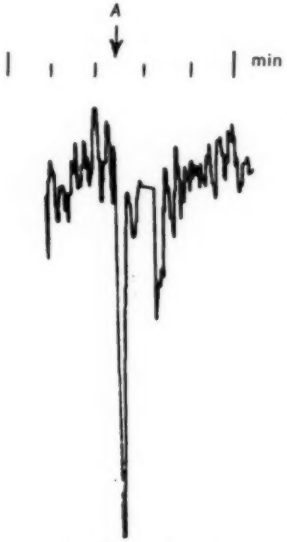

Fig. 2. The photocell record in all the figures illustrates the change in the amount of light transmitted by the pancreas. A downward deflexion is caused by decreased light transmission (i.e. vasodilatation—see text) and an upward deflexion by increased light transmission (i.e. vasoconstriction). At *A* acetylcholine bromide 10 μg was injected arterially. No secretion was observed.

Histamine. Intravenous injection of histamine acid phosphate 5–10 μg caused vasodilatation lasting 1–2 min, but no pancreatic secretion. This is shown in Fig. 3.

Secretin. Arterial or intravenous injection of secretin caused an immediate vasodilatation which preceded the onset of secretion and was usually complete within 5 min. There was no change in the arterial blood pressure. The response, which is illustrated in Fig. 4, was unaffected by atropine or mepyramine in doses which abolished the effects of acetylcholine and histamine respectively.

In view of the finding of Bennett & Still (1933), that increase in blood flow in the secretin-stimulated pancreas only occurred if there was obstruction to outflow of juice, it was necessary to find out if the vasodilatation observed in response to secretin was due to a rise in pressure inside the ducts. The following observations show that it was not.

(*a*) Clamping the pancreatic duct cannula after injecting secretin, either before secretion occurred, or during secretion, did not cause any greater vasodilatation than was produced by secretin alone (Fig. 5*a*).

(*b*) Injection of saline into the pancreatic duct at a rate similar to the rate of secretion of pancreatic juice did not have any effect upon the blood content (Fig. 5*b*).

(*c*) In some experiments the pancreatic duct was not cannulated and the juice was free to flow into the duodenum. The vasodilator response to secretin was still recorded under these conditions, although it was very unlikely that there was obstruction to the free exit of pancreatic juice (Fig. 6).

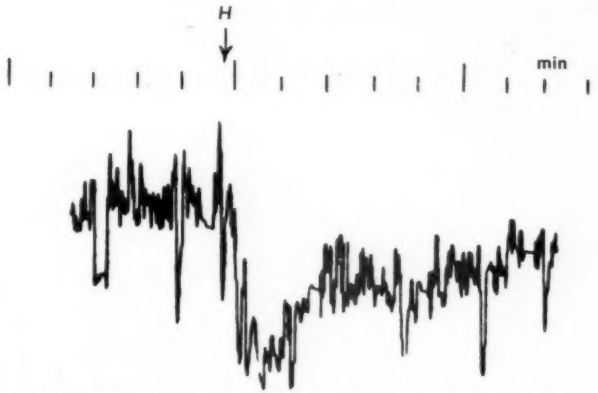

Fig. 3. The vasodilator effect of 10 μg histamine acid phosphate (*H*) injected intravenously. No secretion was observed.

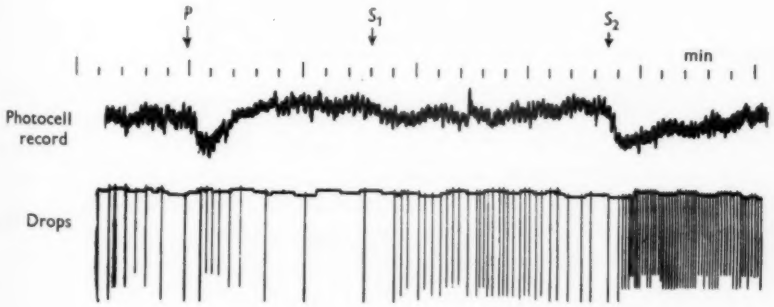

Fig. 4. The vasodilator effect of intravenous injection of 3 u. pancreozymin (*P*), 3 u. secretin (S_1) and 6 u. secretin (S_2). The bottom records in this figure and in Figure 5*a* show secretion of pancreatic juice; each vertical line corresponds to one drop; there is no significance in the different lengths of the lines.

Pancreozymin. Injection of 3–10 units of pancreozymin produced vasodilatation like that produced by secretin, and similarly resistant to atropine and mepyramine. This is shown in Fig. 4, which also illustrates that on this occasion the pancreozymin produced a small increase in the rate of secretion of juice, possibly due to the presence of small amounts of secretin. But this increase in rate was not always observed.

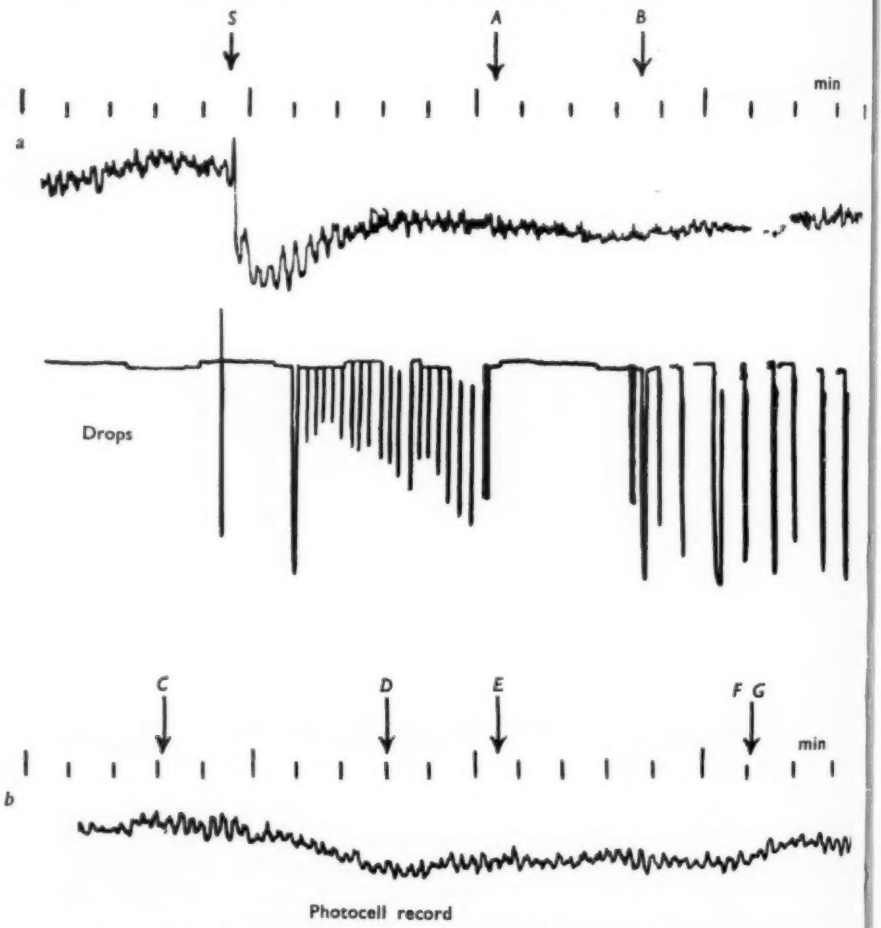

Fig. 5 (*a*). The vasodilator effect of 3 u. secretin given intravenously at *S*. Between *A* and *B* the cannula was clamped to obstruct the flow of pancreatic juice.
Fig. 5 (*b*). This photocell record was obtained from the same experiment as Fig. 5 (*a*). 0·15 ml. saline was injected backwards into the pancreatic duct between *C* and *D* and again between *F* and *G*; the duct was clamped from *D* to *E*.

DISCUSSION

The photocell method measures changes in the opacity of the portion of the pancreas under observation. In the foregoing account we have assumed that these changes were due to an alteration in the amount of blood in the minute vessels. They could, however, be due to changes in the amount of colourless fluid in the gland (pancreatic juice or tissue fluid) or to alterations in the numbers of secretory granules in the cells.

The effect of changes in the amount of colourless fluid in the gland was studied by injecting saline into the blood supply and into the duct system. The photocell was affected only when the blood in the vessels was replaced by intra-arterial injections of saline, and did not respond to back injection of saline into the pancreatic duct, nor to obstruction to the outflow of pancreatic juice.

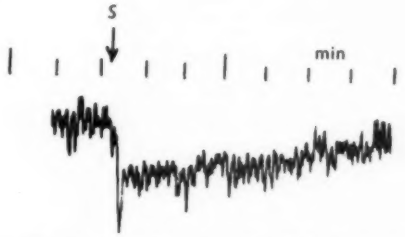

Fig. 6. The vasodilator effect of 2 u. secretin (*S*) in a preparation in which the pancreatic duct was not cannulated.

Histological observations by Harper & MacKay (1948) have shown that pancreozymin causes a decrease in the numbers of secretory granules in the cells: this would cause a decrease in opacity of the tissue instead of the increase observed in our experiments.

It can be concluded, therefore, that the changes in opacity which were observed were not due to changes in the amount of colourless fluid in the gland, nor to changes in granularity in the cells, and it is justifiable to assume that they were due to vasodilatation and vasoconstriction.

Both secretin and pancreozymin produced an increased blood content in the pancreas. Our results with secretin accord with those of Gayet & Guillaume (1933), who used a plethysmograph which also measures changes in blood content. We cannot, however, conclude from our observations that the vasodilatation is accompanied by an increased blood flow through the pancreas during activity. Although it is likely that dilatation of the capillaries and the resistance vessels would occur together this has not been established. It is interesting that pancreozymin causes vasodilatation, since there is little change in the volume of the juice secreted when this

hormone is injected. It is known, however, from the observations of Davies, Harper & MacKay (1949) that pancreozymin, like secretin, stimulates oxygen consumption of pancreatic tissue *in vitro*. The vasodilatation observed in the intact gland would increase the oxygen supply to the cells stimulated by pancreozymin.

SUMMARY

1. A method is described for measuring changes in the blood content of the pancreas of the anaesthetized cat.

2. Acetylcholine and histamine produce vasodilatation, abolished respectively by atropine and mepyramine. Neither acetylcholine nor histamine altered the rate of secretion of pancreatic juice.

3. Secretin and pancreozymin both cause an immediate vasodilatation lasting 2–5 min, and unaffected by atropine or mepyramine.

4. No evidence is presented relating changes in blood flow through the gland to its activity.

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APPENDIX

BY A. E. LOWE

An amplifier for use with a photocell

In the method described above it was necessary to record small variations in transmission of light by the tissue under observation. The resultant variations in light incident on the photocell produced changes of only a few per cent in the total photocell current. These small changes had to be displayed on the record. An amplifier was therefore required which was capable of supplying a voltage output many times that needed for full-

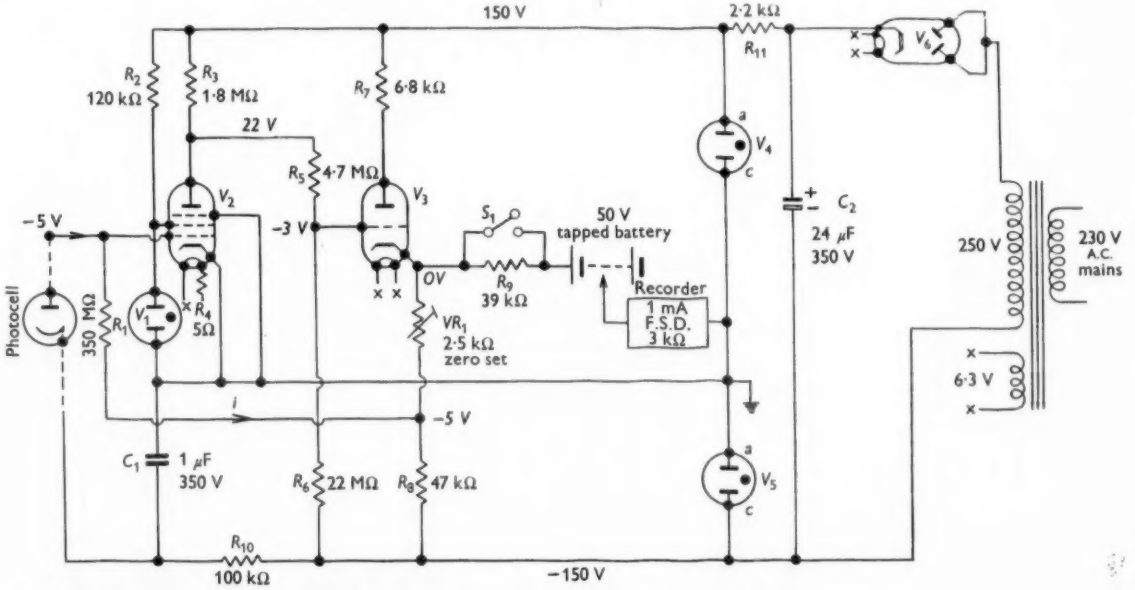

Fig. 7. Circuit diagram of the photocell amplifier. For explanation see text. The voltages shown in italics are the standing potentials when the output is 0 volts ($i = 0$) and vary with the output voltage. Resistors are all 0·5 W, 10 % tolerance, except R_{11} which is 3 W, wire-wound.

Valves:

V_1 CV 2213 (NT 2)	V_4 CV 1832 (OA 2)
V_2 CV 4006 (M 8195)	V_5 CV 1833 OB 2)
V_3 ½ CV 4024 (M 8162)	V_6 CV 493 (6X 4)

scale deflexion of the recorder. If most of this output voltage (corresponding to the resting state of the pancreas) is backed off by an approximately equal voltage of opposite polarity, changes of a few per cent of the total transmitted light can be made to occupy a large proportion of the width of the recorder chart.

The amplifier constructed is a modified feed-back valve voltmeter. It has the following characteristics:

1. The photocell voltage is kept constant, so that the photocell current is linearly related to the incident light.

2. The amplifier output is at least ten times that required for full-scale deflexion of the recorder (3 V). The output is linearly related to the photocell current and independent of the recorder current up to 40 V. The output current is limited to protect the recorder from overloading.

3. The noise and drift of the amplifier is very low.

The circuit diagram is shown in Fig. 7. The photocell current (i) produces a voltage $v = iR_1$ across R_1, and since the grid of V_2 is held at constant potential by negative feed-back (from V_3 via VR_1 to R_1) almost all of v (approximately 99 %) appears as a positive voltage at the output of the amplifier (cathode of V_3). The photocell voltage is therefore held constant, as its anode is connected to the grid of V_2 and its cathode to the negative stabilized line via a smoothing circuit. In practice the maximum value of i is 0·1 μA and as $R_1 = 350$ MΩ the maximum value of the output voltage is about 35 V and the amplifier is linear over this range. As the output valve is a cathode follower with added negative feed-back the output current is independent of the output voltage.

A resistor R_9, between the amplifier and the recorder, reduces the voltage to the recorder for initial setting up (battery at OV) (see Methods). After the backing-off voltage has been found S_1 is closed so that R_9 is short-circuited. It will be seen that the recorder current must pass through R_7 or R_8. These are fixed at values which prevent serious overloading of the recorder.

Low noise and drift are ensured by the following precautions:

(1) V_2 and V_3 are high quality valves of the Ministry of Supply CV 4000 series; (2) R_1 is a high stability component (Welmeg, Welwyn Electrical Laboratories, Ltd.); (3) both positive and negative lines are stabilized (V_4 and V_5) and the screen grid of V_2 is fed from the stabilizer (V_1); (4) R_4 decreases the heater current of V_2, thus bringing its grid current to a level of (10^{-10} to 10^{-11} A) which is very small compared with the photocell currents to be measured.

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CUTANEOUS ANTIGEN-ANTIBODY REACTIONS IN THE RAT

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(*Received* 1 *July* 1959)

In an earlier paper (Brocklehurst, Humphrey & Perry, 1955) we showed that when rabbit antibody was injected into the skin of rats, and antigen administered intravenously, the local rapid increase in capillary permeability (passive cutaneous anaphylaxis, PCA) and the inflammatory changes of the Arthus reaction proceeded normally even in skin depleted of up to 90 % of its histamine content. Furthermore, in normal rats or in rats with greatly reduced skin histamine, antihistamine compounds failed to modify Arthus and PCA reactions unless these drugs were given in such large doses as to produce signs of collapse in the rats. In histamine-depleted animals, treated with an antihistamine drug, the PCA reaction remained unchanged although the amount of histamine required to produce a similar reaction by injection was about 1000 times as great as was present in the corresponding area of skin. We concluded therefore that histamine release, as a consequence of the antigen-antibody reaction, could not be the cause of the observed phenomena.

Inderbitzin & Craps (1957) have confirmed these findings in the rat, and Alberty & Takkunen (1957) found mepyramine to be relatively ineffective against cutaneous anaphylaxis in actively sensitized guinea-pigs. Halpern, Liacopoulos & Briot (1956) have reported, however, that the threshold dose of antigen required to produce a detectable increase in capillary permeability in the skin of actively sensitized rats was increased some 500 times by promethazine (2·5 mg/kg).

Another possible mediator in anaphylactic reactions, besides histamine, is 5-hydroxytryptamine (5-HT), which has been shown to be released by antigen-antibody reactions *in vitro* from blood platelets (Humphrey & Jaques, 1955), and *in vivo* in the rabbit (Waalkes, Weissbach, Bozicevich & Udenfriend, 1957). Parratt & West (1957*b*) showed that rat skin contained 5-HT, but concluded that most of this substance was located elsewhere than in the mast cells, which contained some 90 % of the skin histamine.

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They showed that, although compound 48/80 released both histamine and 5-HT from rat skin, reserpine could greatly diminish the 5-HT while hardly affecting the histamine. Inderbitzin & Craps (1957) reported that bromolysergic acid diethylamide (BOL), a potent antagonist of 5-HT, did not modify the PCA response in rats. Our previous studies had presumably excluded participation of 5-HT in PCA reactions, since the animals were thoroughly treated with 48/80, but this point had not been studied specifically. We have now extended our earlier work by investigating both reversed and direct cutaneous anaphylactic reactions, with particular reference to the effect of antihistamine drugs and BOL, in rats whose skin content of both histamine and 5-HT had been greatly reduced. The results indicate that neither histamine nor 5-HT are primarily involved in these skin reactions.

METHODS

Animals. The rats used in these experiments were drawn from the same colony as were those used in our previous studies (Brocklehurst *et al.* 1955).

Depletion and estimation of skin histamine. The procedures for depleting rat skin of histamine by compound 48/80 and for determining the skin histamine content were those described previously (Brocklehurst *et al.* 1955).

Depletion and estimation of skin 5-HT. The use of 48/80 to deplete the skin of histamine also causes a marked reduction in 5-HT (Parratt & West, 1957*b*). We have also used reserpine as an independent means of reducing the 5-HT content of the skin. Large doses of reserpine produce very severe systemic effects including dehydration, which make the animals wholly unsuitable for investigations of skin reactions. We used doses of reserpine of from 0·5 to 2·0 mg/kg, which did not induce prolonged systemic effects, and, in combination with the treatment with compound 48/80, reduced the skin 5-HT to some 10% of normal. A typical course of treatment is shown in Table 1.

TABLE 1. Typical course of treatment for depletion of skin histamine and 5-HT

	Dose (mg/kg)	
Day	Compound 48/80	Reserpine
1	0·8	—
2	1·2 and 1·4	—
3	1·8 and 2·2	—
4	3·0	—
5	3·8	—
6	5·0	—
7	—	1·0
8	—	1·5
10	—	2·0
14	Animals used for experiment	

The method of estimating the amount of 5-HT in the skin was based on that of Parratt & West (1957*a*). 150–200 mg of shaved skin was taken from areas of the thorax and abdomen, adjacent to the reaction site, and freed from subcutaneous fat. This was dropped into 5 ml. of acetone at 0° C and, after being chopped fine with scissors, was kept at 4° C for 36 hr. The supernatant fluid was then collected, and 2 ml. acetone was added to the tissue, which stood for a further 2 hr. The acetone solutions were mixed, and evaporated to dryness at below 10° C; 0·5 ml. water was then added and the sample was kept at −15° C. The 5-HT

content was estimated on the rat uterus, at 30° C, in Tyrode's solution modified according to Schwartz, Masson & Page (1955) and aerated with 95 % oxygen + 5 % CO_2.

Production of reversed PCA reactions. Reversed PCA reactions were produced as before (Brocklehurst *et al.* 1955). The antigens used were (1) crystallized Bovine Serum Albumin (BSA, Armour Laboratories), (2) Human Serum Albumin (HSA. kindly supplied by Dr W. D'A. Maycock, M.R.C. Blood Products Unit, Lister Institute, Elstree) and (3) hen ovalbumin (EA), recrystallized three times. Rabbit antisera were prepared by repeated intravenous injection of antigen adsorbed on alum. The antibody content, measured by quantitative precipitation at optimal proportions, was 5–10 mg/ml. Rat antibodies were prepared as described below under 'Active sensitization'.

Production of direct PCA reactions. The same antigen–antibody systems were used as for the reversed PCA reaction. In preliminary experiments antibody prepared in rabbits or rats was administered intraperitoneally or intravenously to normal rats in doses of 2–12 mg/100 g body weight. Skin reactions to various amounts of intracutaneous antigen were tested 24 or 48 hr later. We found that when antiserum was administered intraperitoneally, whatever the quantity of antiserum used, there was very little response to intracutaneous antigen. When the antiserum was given intravenously, a graded cutaneous anaphylactic response was obtained over a suitable range of test doses of antigen.

Reproducible graded skin responses of convenient size, and with satisfactory dose–response slope, were obtained when 6 mg antibody/100 g was administered intravenously, and the skin test doses of antigen were 0·5–3 μg (in 0·05 ml.). The slope of the dose–response curve was less steep than with PCA reactions, and became very flat at higher doses of antigen.

Active sensitization. Rats are not readily sensitized by the usual techniques, but a method based on that of Lipton, Stone & Freund (1956) was successful. BSA, HSA or EA was incorporated at a concentration of 10 mg/ml. in an oil-in-water emulsion containing killed tubercle bacilli. Two doses of 0·5 ml. were injected intramuscularly one week apart. Five weeks after the second injection 1 mg antigen adsorbed on aluminium hydroxide was administered intravenously, and a further similar injection was given a week later. The second intravenous injections caused temporary prostration (anaphylatic shock), and the precipitating antibody levels subsequently rose to 1·5–7 mg/ml. Ten to twelve days after the last injection the rats were used for tests of active cutaneous anaphylaxis, or were bled in order to provide rat antibodies for use in PCA tests using homologous serum.

Design of experiments. The experimental designs, as before (Brocklehurst *et al.* 1955), included randomization of injections and blind reading of results. Histamine acid phosphate and 5-HT creatinine sulphate were made up as aqueous solutions containing 1 % base, and the pH was adjusted to 7·5 with N/3-NaOH. Subsequent dilutions for intradermal injection were made with Tyrode's solution.

RESULTS

Effect of compound 48/80 *and reserpine on content of 5-HT in rat skin*

After the combined course of treatment with compound 48/80 and reserpine described above, the content of 5-HT in the rat skin was found to be reduced by about 90 % at the time when the cutaneous anaphylactic reactions were produced. The results of one experiment showing the extent of variation from animal to animal are given in Table 2.

In most experiments the skin samples (from various groups) were pooled before assay. The mean values were similar. The skin histamine content of these animals at this time of performing the anaphylactic reactions was not more than 15 % of that in control rats. These results agree with the figures

given by Parratt & West (1957*a*) and by Bhattacharya & Lewis (1956). The animals remained in good condition at the end of the course of treatment.

TABLE 2. The effect of treatment with compound 48/80 and reserpine on the 5-HT content of rat abdominal skin. (For dose schedule see Table 1.) The values are μg base/g skin

Control animals	0·66, 0·79, 1·4, 1·56	Mean 1·10
Treated animals	0·1, 0·1, 0·12, 0·15 0·1, 0·1, 0·16, 0·12	Mean 0·12

The effect of antihistamine drugs and BOL on reversed PCA reactions

Both in rats with low skin histamine and 5-HT and in control rats the responses to intracutaneous injections of histamine and 5-HT were used as an index of the effectiveness as inhibitors of mepyramine and BOL. The results are shown in Fig. 1, in which three experiments are combined. It will be seen that BOL (2 mg/kg), freshly prepared and injected intravenously as a 0·2 % aqueous solution about 30 min before the tests were made, reduced the skin response to injected 5-HT somewhat more than fiftyfold. In the skins of rats depleted of 5-HT by combined treatment with compound 48/80 and reserpine, the responses to intracutaneous 5-HT (but not to histamine) were somewhat larger, and the effect of BOL was to reduce these responses even more than in control rats. This may be related to the fact that the skin of rats treated with 48/80 is very thin, and the distribution of an injected dose will consequently be modified. BOL did not diminish the size of the responses to injected histamine, but they were distinctly paler than in control rats. Mepyramine (50 mg/kg intraperitoneally 45 min before testing) reduced the response to histamine from sixty to two hundred times in this and other experiments, without significantly affecting the response to 5-HT.

Reversed PCA reactions were carried out in control rats and in rats pretreated with compound 48/80 and with reserpine, with and without mepyramine and BOL treatment. The results are shown in Fig. 2. The mean diameters of the PCA lesions were very similar in all groups, the greatest variations observed corresponding to less than a twofold increase of dose. It would seem therefore that neither histamine nor 5-HT, separately or together, determine the size of the lesion which results from reversed passive anaphylactic reactions in the rat skin. There were differences, however, in the intensity of the reactions. Rats treated with BOL gave PCA reactions notably paler than rats not so treated. This was observed in rats with normal or greatly diminished skin histamine and 5-HT.

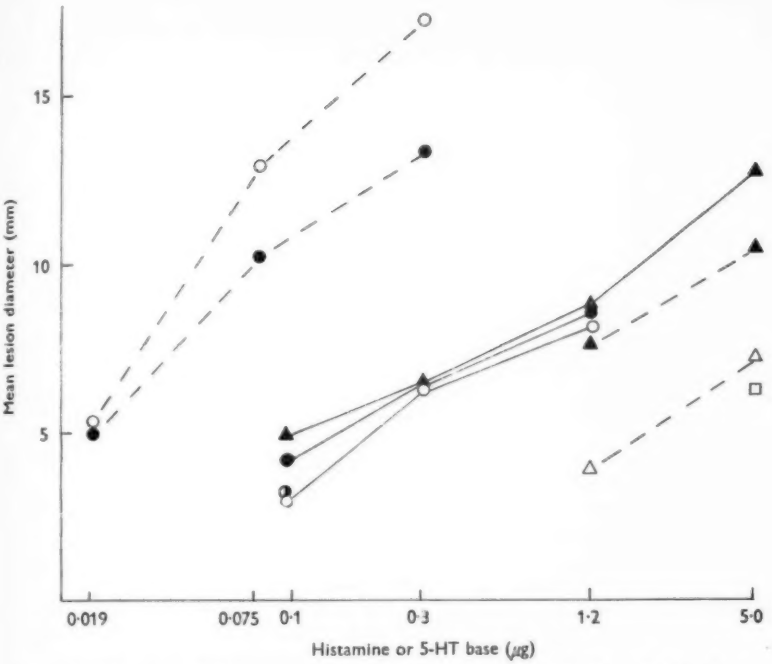

Fig. 1. Diameter of blue areas produced by intracutaneous injection of histamine and 5-HT in normal and treated rats, in the presence and absence of BOL. Filled symbols denote normal rats, open symbols denote rats treated with 48/80 and reserpine: ◑, treated with reserpine only; ●○, no antagonist; △▲, BOL; □, BOL + mepyramine; —— = histamine response; – – – = 5-HT response. Semi-log. scale. In this and subsequent figures each point represents the mean response in 4–10 animals.

Direct cutaneous reactions in actively and passively sensitized rats

When direct PCA reactions were elicited by injection of antigen into the skin of rats passively sensitized with rabbit or rat antibody, the area of the response was not diminished significantly by either mepyramine or BOL, but the intensity of blueing was reduced by BOL. This was particularly evident in the smaller lesions, which were sometimes too pale to be measured. The results of one experiment are given in Table 3.

The combined results of two experiments with rats which had been actively sensitized are shown in Fig. 3. The doses of the inhibitors tested were sufficient to depress the responses to intracutaneous histamine and 5-HT by a factor of well over 100, yet the response to antigen was reduced only twofold by mepyramine, and BOL had no effect on the area and a

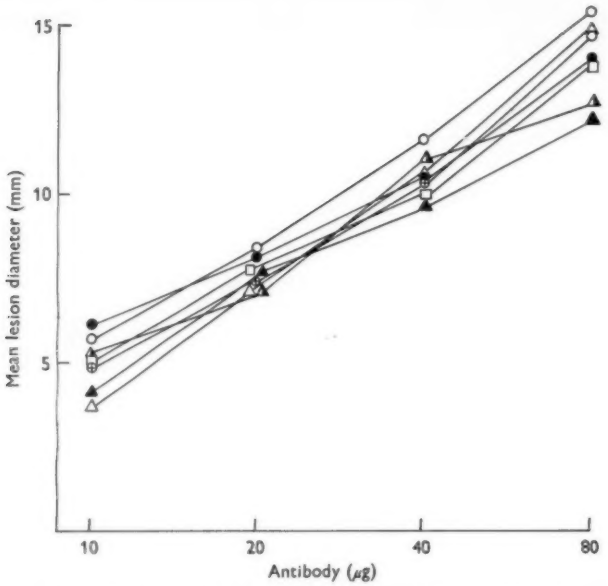

Fig. 2. Diameter of PCA reactions in normal and treated rats in the presence and absence of BOL and/or mepyramine. Filled symbols denote normal rats, open symbols denote rats treated with 48/80 and reserpine: ●○, no antagonist; △▲, BOL; □, BOL + mepyramine; ⊕, treated + mepyramine; ▲, reserpine + BOL. Semi-log. scale.

TABLE 3. Direct cutaneous anaphylaxis in passively sensitized rats. Mean lesion diameter (mm). Intensity of blue indicated on a scale of + to + + + + +

	Antigen (μg)			
Antagonist	1/8	1/2	2	8
None	4·6 + +	6·8 + + +	9·8 + + +	11·6 + + + +
Mepyramine	3·2 + +	6·6 + +	9·1 + + +	11·0 + + + +
BOL	5·0 +	6·0 +	8·5 + +	10·4 + + +
BOL + mepyramine	Unreadable ±	6·0 +	9·1 + +	12·0 + + +

Antigen given intradermally in 0·05 ml. Tyrode solution 44 hr after antibody given I.V. The antagonists reduced the response to 5-HT and histamine by a factor of at least 100.

very slight effect on the intensity of the lesions. Promethazine (20 mg/kg) reduced the size of the lesions produced by antigen, in that, after promethazine, 5–10 times more antigen was required to elicit responses similar to those in untreated rats.

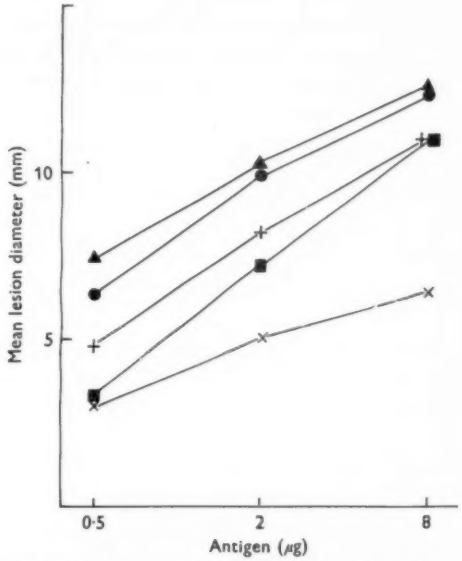

Fig. 3. Diameter of direct cutaneous anaphylactic reactions in actively sensitized rats. The rats were actively sensitized with HSA or EA, and had not been treated with 48/80 and reserpine. Both antigen–antibody systems gave comparable results. ●, no antagonist; ▲, BOL; +, mepyramine; ■, BOL+mepyramine; ×, promethazine. Semi-log. scale.

The effect of promethazine on other cutaneous reactions

Promethazine was the only antihistamine drug significantly to affect cutaneous anaphylactic reactions in this and in our previous series of experiments. Benacerraf & Fischel (1949) found that in the rabbit the Arthus reaction and the effect of an erythrogenic toxin from streptococci were markedly inhibited by comparable doses of promethazine, and concluded that this drug acts directly on capillaries, reducing their susceptibility to damage. Furthermore, promethazine (20 mg/kg), unlike mepyramine, diminished the skin response of rats to 5-HT by about eightfold, in addition to more than a thousandfold reduction of the skin response to histamine.

It was therefore interesting to examine the effect of promethazine on the action of the permeability factor of guinea-pig serum described by Wilhelm,

Miles & Mackay (1955). This factor is activated by dilution or ether fractionation of serum, and is concentrated in the G 2α globulin, a sample of which was kindly provided by Professor A. A. Miles. The factor has been shown by Wilhelm and his colleagues to act independently of histamine, and not to be a histamine liberator in the rat (Wilhelm, Mill, Sparrow, Mackay & Miles, 1958). The effect of promethazine (20 mg/kg) and mepyramine (50 mg/kg) on the areas of increased capillary permeability due to graded doses of the G 2α fraction of guinea-pig serum is shown in Fig. 4.

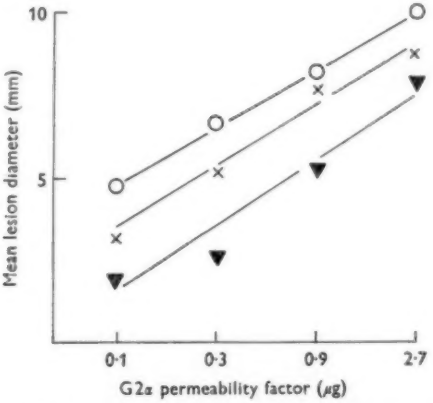

Fig. 4. Diameter of skin reactions to guinea-pig G 2α permeability factor. The material was injected in 0·05 ml. Readings were made on the under side of the skin 25 min after injection. ○, no antagonist; ×, mepyramine 50 mg/kg; ▼, promethazine 20 mg/kg. Semi-log. scale.

It is interesting to note that mepyramine diminished the response about twofold, since Wilhelm *et al.* found that rat permeability factor, tested in the rat, was also weakly inhibited by mepyramine. Promethazine, however, diminished the response some sixfold and affected not only the area but the intensity of the weaker responses, which were relatively pale and faded at the edges.

DISCUSSION

We have extended our earlier observations on reversed PCA reactions to include the use of both heterologous and homologous antibodies and also experiments on direct cutaneous anaphylaxis in actively and passively sensitized rats. In no case have we been able to show that depletion of the skin 5-HT and histamine affects the size of the cutaneous anaphylactic reaction, even when the cutaneous reaction to injected 5-HT and histamine is inhibited with BOL and mepyramine. Two points require some amplification, however. First, BOL definitely reduced the intensity of blueing in

the area of the lesions produced both by intradermal injections of histamine and by cutaneous anaphylactic reactions. Furthermore, in skin depleted of both histamine and 5-HT, mepyramine also decreased the intensity of the anaphylactic reactions. It is known that histamine is released in these reactions and although there is no direct evidence for a release of 5-HT, the effect of BOL suggests that this also occurs. Both histamine and 5-HT therefore may play some part, almost certainly secondary, in increasing the intensity of anaphylactic reactions in rat skin. The underlying mechanism, however, appears not to be dependent on them, a conclusion already reached by Inderbitzin & Craps (1957) and by Sanyal & West (1958). The only tissue reaction which appears to be mediated principally by 5-HT is the anaphylactic contraction of the mouse uterus (Fink & Rothlauf, 1955); this must not be taken to apply generally, since one of us (Brocklehurst, 1958) has shown that the anaphylactic contraction of the rat uterus cannot be suppressed by antagonists of 5-HT.

Secondly, attention is drawn to the action of promethazine. This is the only antihistamine drug which we have found to affect significantly the size of the cutaneous anaphylactic reaction, and Halpern *et al.* (1956) found it even more potent in raising the threshold of this response. This action of promethazine seems not to depend on its antihistamine properties but upon some other non-specific effect on capillaries which is not shared by mepyramine. Inhibition by promethazine is much less obvious in tests with reversed PCA than in direct cutaneous anaphylactic reactions. Halpern *et al.* came to similar conclusions, and have doubted whether reversed PCA reactions should be classed as true anaphylactic reactions. In an appendix to this paper the mechanisms of both reactions are analysed, in order to explain why apparently contradictory results may be obtained with inhibitors, and why reversed PCA reactions are more suitable than direct reactions for the study of the underlying processes of anaphylaxis.

One other point requires discussion, namely the difference in the relation between dose and diameter of skin reactions to injected histamine and to 5-HT. These amines are similar in molecular size; neither binds very markedly to tissues; and the responses of both were measured after 20 min. The slope of the line relating log. dose to response might therefore be expected to be similar also. In fact the slope with 5-HT was the steeper, which would be explained if, in the skin, histamine were inactivated enzymically more rapidly than 5-HT; this explanation receives some support from the observations of Vane (1959) that 5-HT appears to be resistant to the amine oxidases of intact rat stomach preparations whereas amines lacking the –OH group are not.

SUMMARY

1. Reversed passive cutaneous anaphylactic reactions, and direct cutaneous anaphylactic reactions have been studied in passively and actively sensitized rats. The effect upon these reactions of depleting the skin of histamine and 5-HT by compound 48/80 and/or reserpine, and of treatment with mepyramine and/or BOL, has been examined.

2. No evidence was found for significant effects of any of the treatments, alone or in combination, on the size of the anaphylactic responses, although in rats treated with BOL they were paler.

3. Promethazine reduced the anaphylactic responses, but reasons are given for considering this effect to be due to some property other than its antihistamine action.

4. It is concluded that histamine and 5-HT are not primary mediators of the anaphylactic reaction in the rat skin, but play a secondary part.

5. The differences between reversed and direct cutaneous anaphylactic reactions are considered to be due to the mechanical factors determining the spread of antigen which reacts with antibody, and reasons are given for preferring the former for study of antigen–antibody reactions of this kind.

We wish to thank Professor A. A. Miles for the sample of G 2α guinea-pig permeability factor; Messrs Ciba for reserpine; Messrs Burroughs Wellcome for 48/80; Abbott Laboratories for 5-HT; Sandoz for Brom LSD; Specia for promethazine; and Messrs May and Baker for mepyramine.

APPENDIX

Comparison of direct (active and passive) and reversed passive cutaneous anaphylactic reactions

When any locally active agent is injected into, or produced in, the skin, the size of the response will depend on the area over which a threshold concentration is reached and maintained long enough for the response to appear. The mechanical factors involved in intracutaneous injections in the guinea-pig have been discussed by Miles & Miles (1952); in the rat skin, which is thinner, the process is probably similar, but the fluid mass will be retained at the injection site for a shorter time. Spread of injected material probably occurs by a combination of mass movement of fluid in the connective tissue, and, to a lesser extent, by diffusion. It will be limited by any process which inactivates the material, and by adsorption on to tissues. The areas over which a response is produced by two different concentrations of the agent have been found experimentally to be proportional to the log. dose. Rate of spread is therefore proportional to the amount present, resembling in this respect processes controlled mainly by diffusion. A mathematical treatment of such processes has been given by Humphrey & Lightbown (1952), which predicts that the slope will be increased the longer the interval between injection of the test material and the reading of the reaction.

In the case of reversed PCA the injected antibody spreads unimpeded in the skin, except for slight adsorption, during 3–4 hr before antigen and indicator dye are administered intravenously. There is evidence that antigen–antibody combination is most damaging when antigen is present in moderate excess (Rosenberg, Chandler & Fischel 1958; Trapani, Garvey & Campbell, 1958). Since, in PCA, excess antigen is injected, this condition is

reached rapidly and simultaneously over the whole area and produces a reaction wherever antibody is present at a supraliminal concentration. A steep slope therefore results. In direct anaphylactic reactions, on the other hand, when graded doses of antigen are given in a fixed volume intracutaneously, they reach only a limited area of skin and there the reaction begins at once. The skin contains a uniform concentration of antibody, and, depending on this concentration and the amounts of antigen used, much of the antigen will combine with the antibody at the injection site, and will not be free to spread over a wider area. Only where there is excess of antigen will the chain of events leading to increased capillary permeability be started, but a large excess of antigen will not necessarily be more effective than a moderate excess. Furthermore, the reaction is read after 25 min, which is a very short time for any antigen remaining uncombined to spread over a wider area and enlarge the lesion. These are conditions which will produce a small slope.

In studying passive direct cutaneous anaphylactic reactions we found that suitable doses of antigen were related to the amount of antibody given, and that the range was rather critical. In the lower dose ranges the slopes of the dose–response curves were sometimes very small, while at higher dose levels the curves showed a plateau. With still higher doses the responses consisted only of a blanched central area with a surrounding indefinite ring of blue. Furthermore, antibody levels vary quite widely in a group of actively sensitized animals; the same dose of antigen may therefore not be optimal for all the animals in the group. For this reason passive sensitization is to be preferred for quantitative work, although it perhaps resembles natural sensitization less closely than does active sensitization. The use of a threshold reaction (in which the end point is the smallest dose of antigen able to produce a detectable lesion) has severe limitations because it gives no indication of the relation between dose and response. An agent such as promethazine, which in our experiments reduced the vascular response to various different agents several fold, apparently non-specifically, could raise the concentration of antigen needed to produce a threshold reaction by a large factor, as indeed was found by Halpern *et al.* (1956). However, there need be no quantitative relation between this increased threshold and, on the one hand, the specific inhibition of the anaphylactic reaction, or, on the other, the antagonism of substances acting directly, such as histamine and 5-HT. For example, if the level of sensitization were such that over a wide range of antigen test doses the effect of the antigen–antibody reaction on capillary permeability exceeded the threshold by a relatively small amount, a non-specific agent capable of raising the threshold by this amount would completely prevent the skin from showing a response, irrespective of the dose of antigen.

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THE EXCITATORY ACTION OF ACETYLCHOLINE ON CUTANEOUS NON-MYELINATED FIBRES

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Some idea of the sensory pathways involved in signalling pain might be obtained from studying the pattern of sensory discharge in the different groups of sensory nerve fibres set up by pain-producing drugs. Some forms of pain are thought to be mediated by small myelinated fibres, and fibres of this type in cutaneous nerves are known to be excited by acetylcholine (Brown & Gray, 1948; Douglas & Gray, 1953)—a drug which causes pain when injected into the skin (Armstrong, Dry, Keele & Markham, 1953). It would be valuable to know whether the action of acetylcholine (ACh) was confined to myelinated fibres or whether it extended to the non-myelinated fibres which are also believed to signal certain painful stimuli. This has been the main object of the present experiments. Preliminary accounts of some of our findings have already been given (Douglas & Ritchie, 1957*b*; Douglas & Ritchie, 1959).

METHODS

The technique used to determine the presence of sensory discharges in non-myelinated fibres has already been described in detail (Douglas & Ritchie, 1957*a*, *c*). It depends on the setting up of an evoked antidromic compound action potential and measuirng how this potential is modulated by any orthodromic sensory discharge in the nerve. The more orthodromic sensory impulses the C fibres are carrying, the more likely it is that some of the descending antidromic impulses (which make up the C component of the recorded antidromic compound action potential) will be extinguished in the stretch of nerve lying between the stimulating and recording electrodes by ascending orthodromic impulses. During sensory activity in C fibres the size of the recorded C potential is reduced, and the extent of the reduction can be taken as a measure of the number of C fibres involved and their intensity of discharge.

The experiments were carried out on cats anaesthetized with chloralose (80–100 mg/kg) after induction with ethyl chloride and ether. The details of the methods of electrical recording have been described already (Douglas & Ritchie, 1957*c*). A cat's saphenous nerve was exposed and cut high in the thigh. A pair of stimulating electrodes (*S*) was placed near the cut peripheral end of the nerve and a pair of recording electrodes (*R*) was placed some 70–100 mm away on the fine branch of the nerve supplying a small area of skin about 10–15 cm² lying below the knee (Fig. 1). Compound action potentials were evoked by applying supramaximal electrical shocks of about 0·5 msec duration, regularly, 3 times

every 2 sec through a stimulus isolation unit. The recording electrodes measured both the orthodromic sensory discharge from the skin and the antidromic compound action potential.

Drugs were injected close arterially into the area of skin through an indwelling cannula (28 gauge needle) in the saphenous artery (Fig. 1) by the method described by Brown & Gray (1948). The drugs used (acetylcholine chloride, hexamethonium chloride, atropine sulphate and phentolamine hydrochloride) were all given in Locke's solution (mM: NaCl 154; KCl 5·6; $CaCl_2$ 2·2; $NaHCO_3$ 1·9; dextrose 5). Each injection was 0·2 ml. in volume and took 1–2 sec to complete.

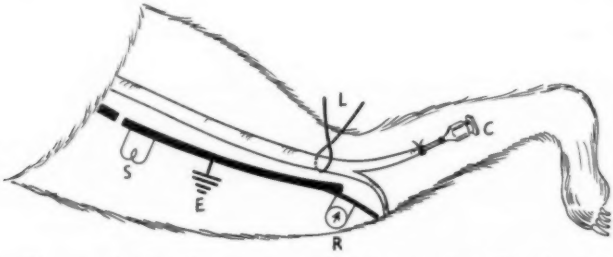

Fig. 1. Diagram showing the arrangement of the recording (*R*), stimulating (*S*) and earthing (*E*) electrodes together with the cannula (*C*) through which close arterial injections were made and the ligature (*L*) used to occlude the main artery during such injections. The recording electrodes are on the branch of the saphenous nerve supplying an area of skin between the knee and the ankle. This area was also supplied by the branch of the superficial femoral artery into which the injections were made.

RESULTS

At the beginning of any experiment the C potential usually had the shape of a single wave with notches in it, presumably corresponding to the different fibre groups described by Gasser (1950). But after stimulation had progressed for a few minutes, the C potential separated into two main components, as had been noted earlier (Douglas & Ritchie, 1957*c*), presumably because slight slowing of conduction occurred in one group of fibres; there was no further change in the configuration of the C potential even though stimulation was continued for hours. For convenience we have called these two components the C_1 and C_2 elevations. Usually the faster component, the C_1 elevation, was by far the larger. The C_1 fibres conducted at about 1 m/sec at 37° C and the C_2 fibres some 10–20 % more slowly.

The effect of acetylcholine on the C potential

When ACh was given by close arterial injection into the skin, there was an immediate outburst of nervous activity lasting several seconds in the branch of the saphenous nerve supplying the injected area. Records of the compound action potential showed that many non-myelinated fibres contributed to the discharge, because for some time after the injection of ACh the C elevation of the compound action potential was greatly reduced

in size. The main effect is seen in Fig. 2*a*. In Fig. 2 the three lower records are control records of the compound action potential and show the C_1 and C_2 elevations; the upper records show how these elevations were affected by various close arterial injections. The upper record in Fig. 2*a* was taken about 1 sec after the beginning of the close arterial injection of 20 μg ACh. Both the C_1 and C_2 elevations are reduced in size, indicating that both the C_1 and C_2 fibres were excited by the drug.

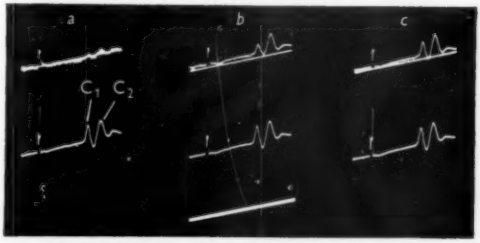

Fig. 2. The effect of acetylcholine on the two main groups of non-myelinated fibres in a branch of the cat's saphenous nerve. The records are of the antidromic compound action potential and mainly show the C_1 and C_2 elevations; the A elevations are largely lost in the stimulus artifact. The lower of each pair of records is the control. The upper records were taken about 4/3 sec after the close arterial injection, into the area of skin supplied by the nerve branch, of (*a*) 20 μg ACh, (*b*) 2 μg ACh, (*c*) Locke's solution. Temperature of the nerve trunk, 34° C; conduction distance 89 mm; time calibration, 200 msec.

Although both main groups of C fibres fired in response to ACh, the C_2 fibres proved less sensitive. This was clearly seen in experiments using smaller doses of ACh. As can be seen in Fig. 2*b*, taken from the same preparation as Fig. 2*a*, 2 μg ACh caused a large fall in the C_1 elevation but only a small fall in the C_2 elevation. On some occasions (Douglas & Ritchie, 1957*b*) it was possible to excite most of the C_1 fibres with ACh without affecting the C_2 fibres.

In these experiments the injection of Locke's solution often produced some discharge in the C_1 fibres, as can be seen in Fig. 2*c*. The discharge was small and never obscured the greater effects produced by ACh. It was presumably a mechanical effect, for many C_1 fibres are mechanosensitive (Douglas & Ritchie, 1957*c*). No discharge was ever detected in C_2 fibres as a result of the injection of Locke's solution.

A comparison of the responses of C_1 and C_2 fibres to acetylcholine

The discharge in the C_1 fibres appeared during the injection, reached a maximum within 1–2 sec of the beginning of the injection and slowly passed off. The sequence of events is illustrated in Fig. 3, which shows

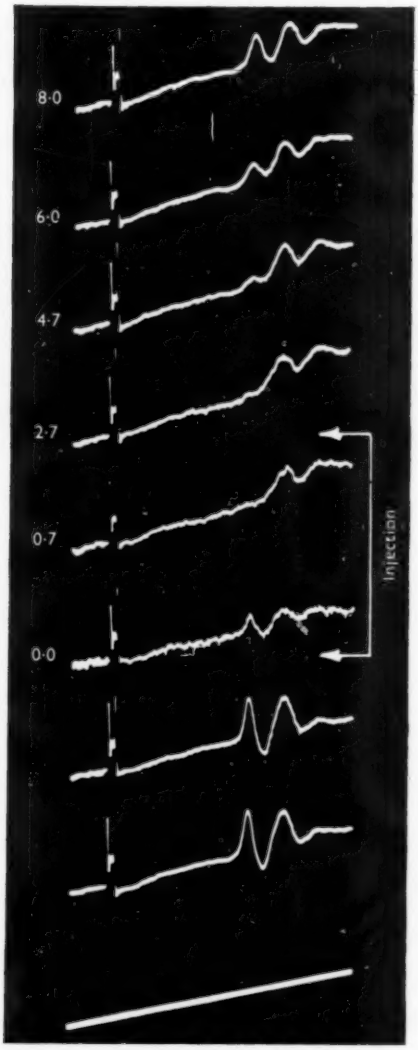

Fig. 3. The time course of the excitatory effect of ACh on the C fibres in a branch of the cat's saphenous nerve. The records, of the antidromic compound action potential, were taken from a continuous series of records obtained every 2/3 sec. The lower two records are the pair taken immediately before the close arterial injection of 20 μg ACh into the area of skin supplied by the knee branch. The arrows indicate the beginning and the end of the injection. The numbers at the side of each record indicate the time in seconds after the beginning of the injection at which each was taken. Temperature of the nerve trunk, 31·5° C; conduction distance, 86 mm; time calibration, 200 msec.

records of the compound action potential selected from a continuous series taken before, during, and after the injection of 20 μg ACh. The effect on the C_2 group of fibres was much more transient. The maximal effect again occurred soon after the beginning of the injection; but some recovery usually occurred even before the injection was over, and complete

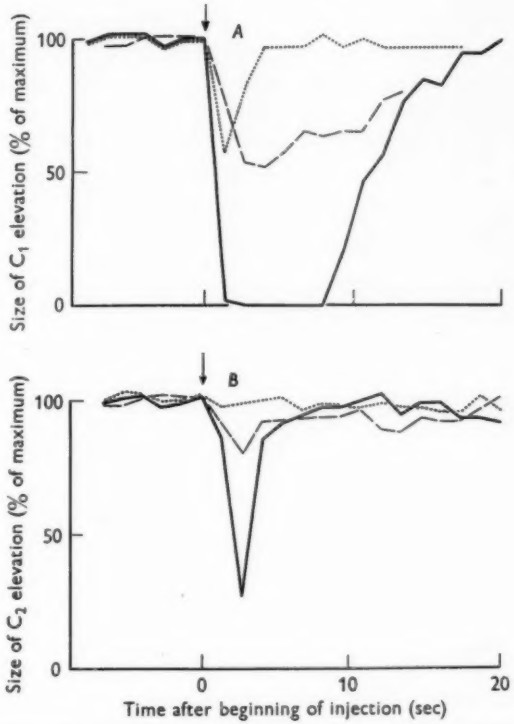

Fig. 4. A comparison of the effects of ACh on the C_1 and C_2 fibres in a branch of the cat's saphenous nerve. The graphs in *A* show the time course of the effect on the C_1 fibres and the graphs in *B* that on the C_2 fibres when saline (dotted line), 2 μg ACh (broken line) and 20 μg ACh (continuous line) were given by close arterial injection into the area of skin supplied by the branch. The beginning of injection is marked by the arrow. Temperature of the nerve trunk, 34° C; conduction distance, 80 mm.

recovery appeared to occur within a few seconds, i.e. at a time when the C_1 fibres were still discharging intensely. This difference in the responses of C_1 and C_2 fibres to ACh can be seen clearly when plotted as in Fig. 4, which shows graphically the time course of the effects of the injections of different amounts of ACh on both the C_1 and the C_2 elevations. Even when a

small dose of ACh was used to produce a comparatively small fall in the C_1 elevation (broken line, Fig. 4*a*) the response long outlasted a much greater fall produced in the C_2 fibres by a higher dose of the drug (solid line, Fig. 4*b*). Thus the C_2 fibres are both less sensitive to ACh and respond more briefly to it.

The way in which acetylcholine excites non-myelinated fibres

Fibres in the C_1 group have previously been shown to be excited by gentle mechanical stimulation (Douglas & Ritchie, 1957*c*). Since the skin contains smooth muscle (for example, pilo-erector muscle) it is possible that the discharges seen in the C_1 fibres after injecting acetylcholine were due to the drug exciting smooth muscle and so providing a mechanical stimulus. However, the evidence suggests that this is not the case, but rather that ACh acts directly on the sensory nerves. Thus the response to ACh was found to be little if at all affected by the presence of atropine, whether the atropine was given systemically some time beforehand (1 mg/kg) or whether it was injected close arterially into the skin (200 μg) immediately before the injection of ACh. These results seem to rule out the possibility that a muscarinic action of ACh on smooth muscle is involved. Further evidence against the C_1 fibre discharge being mediated indirectly by an excitant action of ACh on smooth muscle was provided by experiments where it was shown that hexamethonium (200 μg by close arterial injection), which has practically no antimuscarinic effects, completely abolished the response of the C fibres to a subsequent injection of 20 μg ACh (Fig. 5*c*). Hexamethonium itself did not set up any activity (Fig. 5*b*). At the time when the hexamethonium abolished the C_1 fibre response to injected ACh, the response of these fibres to stroking the skin persisted and appeared to be normal (Fig. 5*d*).

There is an alternative way by which ACh could excite smooth muscle to provide a mechanical stimulus to the sensory nerve endings and which would be consistent with the abolition of the effect by hexamethonium. This possibility is that ACh evokes the afferent discharge by exciting sympathetic motor elements in the skin (either peripheral ganglion cells or more probably the motor nerve endings themselves). But this possibility can be excluded, for the excitant effect of ACh on C fibres persisted when the sympathetic system was blocked by an adrenergic blocking agent. Thus, when phentolamine (Regitine) was given intravenously in a dose of 5 mg/kg (which according to Douglas & Gray (1953) is sufficient completely to block the pilomotor response to sympathetic nerve stimulation) the response of the C fibres to ACh remained unaffected. These experiments seem to rule out the possibility that the discharge seen in C fibres after the injection of ACh is secondary to mechanical stimulation.

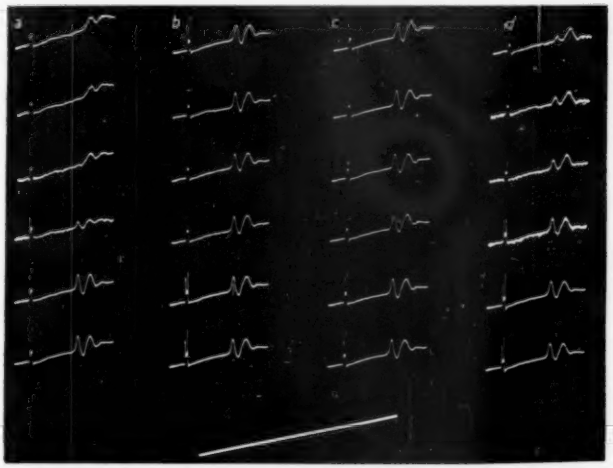

Fig. 5. Antagonism of the excitant effect of ACh by hexamethonium. Four successive tests are shown, each represented by a vertical series of consecutive records of antidromic C potentials evoked every 2/3 sec in a branch of the cat's saphenous nerve. The lowest two records in each series are the controls. Records *a* and *c* show the response to 20 μg ACh given by close arterial injection into the skin before and after 200 μg hexamethonium (given in *b*). Record *d* shows the response to touching the skin, obtained immediately after record *c*.

The response of the different fibre groups to acetylcholine

It is clear that when ACh is injected into the skin, C fibres are excited at least as readily as A fibres. The threshold amount for eliciting a discharge in the most sensitive C_1 fibres was about 2 μg; this is the same as that previously found by Douglas & Gray (1953) for the most sensitive myelinated fibres, and also was the minimal amount observed in the present experiments to produce an outburst of large potentials from the myelinated fibres in conventional standing-spot records taken on moving film (as in Fig. 6) accompanied by a reduction in the α, β and δ elevations of the antidromic compound action potential. When the responses of both the myelinated and non-myelinated fibres to ACh and to touching the skin were compared, it was clear that the chemical stimulus produced a relatively greater discharge in the C_1 fibres and the mechanical stimulus a relatively greater discharge in the large myelinated fibres. Thus, it was possible to abolish the C_1 potential more often in the experiments where the stimulus was touching the skin (as in Fig. 5, where the records show the maximal effects on the C_1 elevation in this preparation to touching the skin and to ACh). On the other hand the stimulus of touching the skin could always produce much more activity in the large myelinated

fibres than could the injection of ACh. Figure 6 shows the barrage of large impulses produced in a typical test of touching the skin; the discharge of large spikes is much greater than could ever be produced even by a large dose of ACh (200 μg). The large spikes, which predominate in conventional records of this type, are derived from the large myelinated fibres.

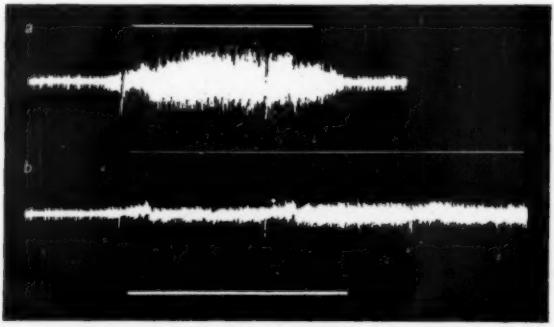

Fig. 6. The different patterns of afferent activity set up by mechanical and chemical stimuli. The records (of a standing spot taken on moving film) show the sensory discharge recorded from the branch of the cat's saphenous nerve supplying an area of skin between the knee and the ankle, (*a*) when the skin was gently stroked (during uppermost horizontal bar), and (*b*) when 200 μg ACh was injected (during middle horizontal bar). The testing antidromic action potentials are seen superimposed: separate records of these taken through shunted amplifiers showed that the C_1 elevation was almost equally affected by both procedures. Lowest horizontal bar, time calibration 1 sec.

The effect of acetylcholine on the after-potential of C fibres

When ACh was given by close arterial injection, a characteristic broadening of the late phase of the recorded C potential was frequently observed during the period following the injection. This effect was sometimes slight, as in Figs. 3 and 5, but often it was quite large, as in Fig. 7. In the experiment of Fig. 7 the records were taken on a slow time base, to show this late effect more clearly. It can be seen that close arterial injection of 200 μg ACh caused the appearance of a large tail to the C elevation. This effect reached a maximum size about 6 sec after the injection and lasted for more than 20 sec. Because of its large amplitude and because it was so much longer in duration than the spike, the late potential could only have been due to the development of some after-potential. It is difficult to analyse such after-potentials in any great detail because the action potentials in these experiments were necessarily recorded diphasically.

Acetylcholine could have produced these after-potentials in two ways. The first possibility is that the after-potentials were secondary to the burst of repetitive activity set up by the ACh exciting the nerve endings, for it is known that repetitive activity may give rise to very large after-potentials in C fibres (e.g. Gasser, 1950; Brown & Holmes, 1956; Ritchie & Straub, 1956). Such after-potential changes, however, have been demonstrated previously only with *electrical* stimulation at *high* rates, and there has been some doubt whether changes in after-potentials can occur in the

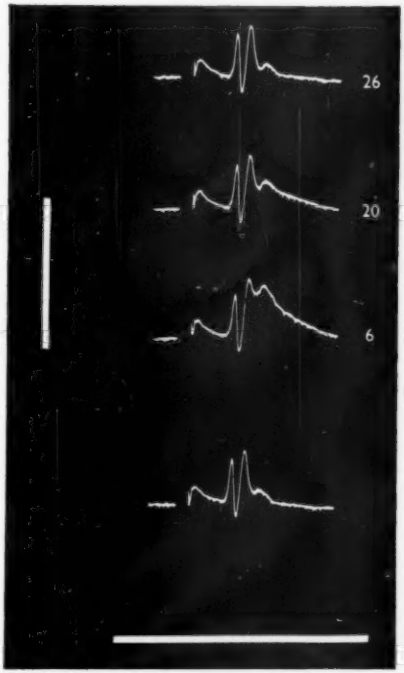

Fig. 7. The effect of ACh on the after-potentials of the C fibres in a branch of the cat's saphenous nerve. The lowest record was taken before and the upper records were taken 6, 20 and 26 sec after the close arterial injection of 200 μg ACh into the nerve–skin preparation. Temperature of the nerve trunk, 30° C; conduction distance 71 mm; time calibration, 500 msec; voltage calibration, 500 μV.

absence of such electrical stimulation (Douglas & Ritchie, 1957*d*). This first possibility implies that under certain conditions the C fibres themselves can in fact generate impulses at a frequency high enough to produce large after-potentials. If this is the mechanism, it is improbable that C_1 fibres are involved, for vigorous discharges set up in these fibres by touching or by cooling the skin (Douglas & Ritchie, 1957*c*; Douglas, Ritchie & Straub, 1959)

are never accompanied by large after-potentials. Moreover, in some experiments after-potentials were produced by ACh which were accompanied by only comparatively feeble activity in C_1 fibres, judging by the relatively small reduction in the C_1 elevation. The second possibility is that the after-potentials arise as a result of a direct action of ACh on the axonal membrane under the recording electrode. In general, the experiments where after-potentials were particularly marked were those in which some of the ACh injected was seen under the microscope to reach at least one of the recording electrodes; the nerve blanched during the injection and tiny droplets of fluid were seen to form on the nerve (the branch of the artery supplying the skin sends minute vessels to the nerve). These latter observations thus give some experimental support for the second possibility, but it is hardly strong enough to be conclusive. We have not yet done the experiments which would allow us to decide which if either of these explanations is correct.

DISCUSSION

Recently Fjällbrant & Iggo (1959) have re-examined the action of ACh on C fibres, but were unable to confirm our finding (Douglas & Ritchie, 1957*b*; Douglas & Ritchie, 1959) of the powerful C fibre discharge to ACh. In their experiments the injections were made into the femoral artery and the amount of ACh which survived dilution and hydrolysis was possibly well below threshold. Circulation through the skin tends to be sluggish and unless the arterial injections are made close to it very little if any of the drug may reach the particular endings being recorded from. In these circumstances, the possibility of overlooking an effect is obviously greater in experiments, such as those of Fjällbrant & Iggo, where single fibres rather than whole populations are being studied. The question is not whether ACh excites C fibres, for this is clearly established by the present experiments, but rather the nature of the C fibres involved. Some of them may be efferent fibres, for according to Heinbecker, O'Leary & Bishop (1933) about 20% of the non-myelinated fibres in the cat's saphenous nerve are sympathetic motor fibres. These might be the fibres responsible for the smaller C_2 elevation, although Gasser (1950) believes that they are not distributed in any single group to give rise to a discrete elevation but contribute to all the elevations of the C potential. Studies on axon reflexes (Coon & Rothman, 1940) have suggested that sympathetic motor fibres in the skin may indeed be excited by ACh. But most of the C fibres responding to ACh are sensory and the bulk of them, the C_1 fibres, are known to be excited by innocuous stimulation such as touching the skin (Douglas & Ritchie, 1957*c*) or cooling it (Douglas *et al.* 1959).

How then may these results be related to the question of pain? One old view is that pain may arise from intense activity in fibres which at moderate

frequencies of discharge signal other modalities. Although this mechanism is not generally accepted (e.g. Adrian, 1931) it has not been rigorously excluded for C fibres. Another view which has sometimes been put forward (see Weddell, Palmer & Taylor, 1959) is that sensation depends on the central analysis of different patterns of activity in populations of afferent nerve fibres set up by different stimuli. Perhaps it is significant that although much of the discharge set up by ACh occurs in populations normally sensitive to innocuous mechanical stimuli, the pattern of afferent discharge set up by ACh is quite different from that set up by touching the skin: thus, for a given degree of activity in the C_1 fibres, touching the skin elicits much more activity in the large myelinated fibres than does injecting ACh. There remains, however, the obvious possibility that there are in the various groups of nerve fibres some specific pain fibres which are excited by ACh along with the mechano-sensitive and thermo-sensitive fibres. Any such specific pain fibres in the C_1 group must be relatively few in number, since the bulk of the C_1 fibres are sensitive to innocuous stimulation. Nor does it seem likely that any large proportion of the C_2 group are specific pain fibres, because it is generally supposed that such pain fibres respond to all forms of noxious stimulation and it has been found that only a small fraction, if any, of this group respond to the classical painful stimulus of heating the skin intensely (Douglas *et al.* 1960). It has already been suggested that the C_2 fibres or many of them may not be sensory at all, but may be sympathetic motor fibres. While the evidence suggests that no more than a small proportion of the non-myelinated fibres in the saphenous nerve can be specific pain fibres, it must be remembered that C fibres are so numerous in this nerve (according to Gasser (1950) they outnumber all the A fibres several times) that even a small *fraction* of them would still be a large *number*.

It seems that ACh causes pain because of an indiscriminate excitant action on nerve endings. Clearly, if more critical information on pain is to be gleaned from studies with drugs, these drugs must be more restricted in their action.

The mechanism and site of action of acetylcholine

The pharmacological analysis we have carried out is essentially that which has previously been used for the myelinated fibres by Brown & Gray (1948) and by Douglas & Gray (1953). Acetylcholine seems to act on non-myelinated fibres *directly*, just as it seems to do on myelinated fibres. The non-myelinated fibres resemble the myelinated fibres in that their sensitivity to ACh is abolished by hexamethonium but not by atropine. Where and how ACh sets up activity either in the myelinated or in the non-myelinated nerve fibres remains unknown.

Many sensory nerves are excited by ACh, and it has often been suggested that ACh plays some part in the initiation of the sensory nerve impulse (e.g. Zotterman, 1953; Liljestrand, 1954). The present experiments offer further evidence against this possibility, for they show that the mechano-sensitive myelinated fibres in the cat's saphenous nerve are like the mechano-sensitive myelinated fibres in the same nerve (Douglas & Gray, 1953), the chemoreceptors in the carotid body (Douglas, 1954) and the baroreceptors in the carotid sinus (Diamond, 1955) in that hexamethonium can completely abolish their sensitivity to ACh without affecting their sensitivity to a physiological stimulus.

It has been argued (Brown & Gray, 1948; Douglas & Gray, 1953) that ACh acts at the nerve *endings*, because of the many experiments reported in the literature where ACh has failed to set up impulses when applied to the nerve trunk; recently, Diamond (1959) has injected ACh intra-arterially into the rabbit's sural nerve *in vivo*, and he has found that it failed to excite fibres of which the axons but not the endings were exposed to the drug. The present experiments, however, suggest that ACh may not be altogether without action on the axonal membrane. Although it may not initiate impulses there, it is possible that it so alters the characteristics of the nerve membrane to cause large after-potentials to appear after the spikes. This latter possibility is of great interest because of the repeated suggestions that ACh may have some part to play in the function of nerve membranes. Such suggestions have suffered from the fact that numerous experiments have always failed to reveal any action of ACh on the nerve membrane. This might be because the preparations used and the particular aspects studied were unsuitable. Because they lack an insulating myelin sheath, and because they are so small and thus have a large surface : volume ratio, mammalian C fibres have proved outstanding material for studying the relationship between metabolism and some of the electrical properties of the membrane (Ritchie & Straub, 1957). It is possible for similar reasons that mammalian non-myelinated fibres are the best material for studying any local action of ACh and it seems worth while to pursue this.

SUMMARY

1. The centripetal activity set up in the cat's saphenous nerve when the pain-producing substance acetylcholine is given by close arterial injection into the skin has been studied.

2. Acetylcholine was found to excite not only large and small myelinated fibres, but also the bulk of the non-myelinated, C, fibres.

3. The C fibres which were most readily excited by acetylcholine belonged to the faster-conducting group, sensitive to touching the skin or to cooling it. A second slower-conducting group of C fibres, for which

no adequate stimulus has yet been found, also discharged in response to acetylcholine. The sensory significance of these discharges is discussed.

4. Acetylcholine seems to excite C fibres, as it does A fibres, mainly by acting directly on them. This sensitivity, although little if at all affected by atropine, was readily abolished by hexamethonium.

5. The touch-sensitive fibres, both myelinated and non-myelinated, continued to respond to touch when their sensitivity to acetylcholine had been abolished by hexamethonium. The normal physiological mechanism for generating impulses in all these mechano-sensitive fibres is therefore not dependent on a sensitivity to acetylcholine.

6. Acetylcholine not only set up impulses in C fibres but also on occasion produced large after-potentials in them. The after-potential changes may be secondary to impulse activity, but may possibly result from some distinct action of acetylcholine on the axonal membrane.

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THE RATE OF FLOW AND COMPOSITION OF VARIOUS SALIVARY SECRETIONS IN SHEEP AND CALVES

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Sheep and cattle secrete large volumes of alkaline and well buffered saliva that serve to stabilize the pH and water content of the fermenting digesta contained in the rumen. A large fraction of this saliva is supplied by the parotid glands, which have been studied by many workers (Colin, 1886; Scheunert & Trautmann, 1921; Coats, Denton, Goding & Wright, 1956; Denton, 1957*b*; Kay, 1958). The parotid glands secrete rapidly and continuously, but the submaxillary glands secrete little except during feeding periods (Colin, 1886; Scheunert & Trautmann, 1921). In addition, considerable quantities of saliva are secreted continuously by the small glands of the mouth (Scheunert & Krzywanek, 1929; Phillipson & Reid, 1958; Kay & Phillipson, 1959) but the glands which contribute to this secretion have not been examined in detail.

Sheep parotid saliva consists of a solution of sodium, potassium, bicarbonate, phosphate and a little chloride (McDougall, 1948), and the relative proportions of these salts are affected by the rate of secretion (Coats & Wright, 1957) and by sodium depletion (Denton, 1956). Relatively little is known of the composition of submaxillary and sublingual saliva or of the secretions of the small glands of the mouth.

More information concerning the non-parotid salivary secretions is required before a balanced knowledge of ruminant saliva can be gained. The present experiments describe the weight and histological appearance of the salivary glands in sheep and calves and provide data on the rate of secretion and composition of parotid, submaxillary, sublingual, inferior molar and palatine saliva.

METHODS

Conscious animals. Suffolk, Cheviot, Blackface and Shetland sheep were used. The Suffolks received hay *ad lib.* and 900–1350 g of mixed linseed and oat meals or flaked maize per day; the other sheep received 900 g of chopped hay and 225 g of mixed meals per day.

Parotid and submaxillary secretions were collected while the sheep were restrained in recording stands. The sheep were accustomed to this procedure, the stands were placed in the sheeps' own pens and the normal feeding routine was continued. The sheep were fitted with rumen cannulae through which the collected saliva or an artificial saliva was returned.

The salivary ducts were cannulated by the method described by Ash & Kay (1959). It

was found that the parotid cannula and collecting tube offered little resistance to the flow of saliva. 15 cm saliva pressure was sufficient to give a flow of over 5 ml./min, the fastest flow normally encountered. The fastest flow of submaxillary saliva, observed during feeding periods, was about 1 ml./min. At 38° C it required a pressure of 60 cm saliva to give this flow of submaxillary saliva through the submaxillary cannula and collecting tube. This is probably higher than the pressure present in the uncannulated submaxillary duct and may have caused some reduction in the rate of secretion.

Anaesthetized animals. Adult sheep and calves aged 5–8 months were anaesthetized by intravenous infusion of 0·9 % NaCl solution saturated with chloralose at 40° C. The parotid ducts were cannulated with polythene tubing immediately behind their papillae, and the submaxillary and sublingual ducts were cannulated beneath the jaw. In some experiments the remaining salivary secretions, which will be called the residual saliva, were collected as they dripped from the mouth and nose. Many of these samples were collected during the experiments reported by Kay & Phillipson (1959). Saliva was stored under liquid paraffin at 1° C until analysed.

The flows of saliva were measured either by volume or by weight. Parotid, submaxillary and residual salivas were collected either in graduated cylinders or in weighed sample bottles. Inferior molar and sublingual secretions were measured either by weight or by recording the flow through graduated pipettes. The mucous secretion of the palatine glands was either aspirated into syringes or was soaked up with weighed swabs which were laid on the palate. In some cases rates of secretion are expressed as g or ml. saliva/g glandular tissue/hr (g/g.hr or ml./g.hr) in order to facilitate comparisons between glands and between animals. Since the specific gravity of saliva is about 1·01 the two expressions are virtually interchangeable.

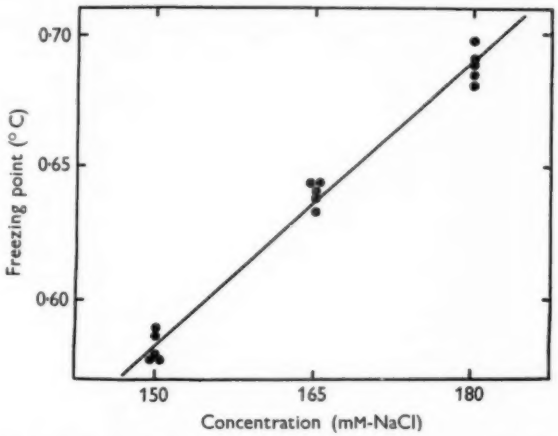
Dissection. At the end of acute experiments the animals were bled from the carotid arteries and the parotid, submaxillary and inferior molar glands removed, freed from extraneous tissue and weighed. In some cases the sublingual glands and the small mucous glands of the mouth were also removed and weighed. The sublingual and palatine glands were dissected easily from the surrounding tissues, but the pharyngeal, buccal and labial glands were largely embedded in muscle and an arbitrary division had to be made, the predominantly glandular tissue being retained and the remainder discarded. This division was most uncertain in the case of the labial glands.

Freezing-point measurements. Measurements were made of the freezing points of plasma and of parotid saliva samples taken from twelve conscious sheep. Two saliva samples were collected under oil, the first taken 5–10 min before feeding and the second while feeding. A sample of blood was drawn from the jugular vein, mixed with a trace of heparin (Liquemin; Roche Products Ltd.) and centrifuged under oil. Two successive measurements of freezing point were made under oil on each sample with a Beckmann thermometer. The second reading was significantly lower than the first by a mean of 0·003° C ($P < 0·05$); this was probably due to loss of CO_2. No correction was made for supercooling; instead, measurements were made of the freezing points of standard NaCl solutions that were allowed to supercool to the same extent as the saliva and plasma samples. The relationship obtained is shown in Text-fig. 1 and was used to convert the observed freezing points to their equivalent NaCl molarity.

Chemical analysis. Sodium and potassium were estimated by flame photometry (Evans Electroselenium). Total CO_2 was estimated by the micro-diffusion technique of Conway (1950) and is expressed as m-equiv HCO_3^-/l. Preliminary measurements of the pH and total CO_2 of sheep plasma and parotid saliva indicated that the CO_2 tension is the same in both fluids, so that in the parotid saliva some 1·2 mM CO_2 will not be ionized. Inorganic phosphate was estimated by the method of Fiske & Subbarow (1925), modified by using the reducing reagent recommended by King (1951). Phosphate concentration is expressed as m-equiv HPO_4^{2-}/l., although in fact the ratio of HPO_4^{2-} to $H_2PO_4^-$ in parotid saliva with a pH of 8·1 (McDougall, 1948) will be 20:1, assuming the pK_2 of phosphoric acid to

be 6·8 (Hitchcock & Taylor, 1938). The proportion of monovalent phosphate will be higher in the less alkaline submaxillary and sublingual secretions. Chloride was estimated potentiometrically (Sanderson, 1952).

Analyses of parotid saliva samples taken from Sheep 3 and Sheep 5 were done singly with few exceptions the remaining analyses were done in duplicate. In 50 duplicate analyses the standard deviation from the means of the duplicates were: Na^+, ±2·5%; K^+, ±2·9% HCO_3^-, ±1·9%; HPO_4^{2-}, ±1·0%; Cl^-, ±2·2%. Where the volumes of saliva available for analysis were very small, as in the case of inferior molar and labial saliva samples, the analytical errors were greater.



Text-fig. 1. The relation between freezing point and concentration of NaCl solutions as measured with a Beckmann thermometer. The regression line has been fitted by the method of least squares.

RESULTS

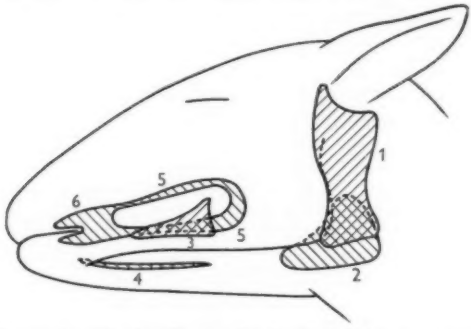
Distribution and weight of salivary glands

The distribution of the larger paired salivary glands of the sheep is shown diagrammatically in Text-fig. 2. A similar distribution was found in the calf. In six sheep and six calves all the salivary glands were dissected out, and their weights are given in Table 1.

The inferior molar glands were wedge-shaped serous glands lying in the cheeks opposite the inferior molar teeth. The glands had many lobes, each of which was drained by a short duct opening on the inferior non-papillated part of the epithelium of the cheek.

The small mucous glands of the mouth constituted a surprisingly large fraction of the total salivary gland mass. The buccal glands were largely confined to the superior and inferior non-papillated parts of the epithelium of the cheek, the inferior group lying beside the inferior molar glands. About half of the palatine glands lay in the soft palate and the remainder in the

posterior part of the hard palate. The pharyngeal glands were fairly evenly distributed over the walls of the oral and laryngeal parts of the pharynx. Mucous glands were found on the lateral margins and root of the tongue and between the tongue and epiglottis and these were included in the pharyngeal group. The labial glands were most numerous at the corners of the mouth. A few scattered mucous glands could be dissected from other parts of the mouth. In the sheep the chief of these were the dorsal sublingual glands and they weighed less than 0·5 g. Thin sheets of mucous and serous glands were found under the epithelium of the nasal cavity, especially near the nostrils and on the dorsal surface of the soft palate.



Text-fig. 2. The main paired salivary glands of the sheep. 1, parotid; 2, submaxillary; 3, inferior molar; 4, sublingual; 5, buccal; 6, labial.

TABLE 1. The weights of the salivary glands in adult sheep and in calves

	6 sheep		6 calves	
	Mean	Range	Mean	Range
Body weight (kg)	43	38–50	102	71–134
Salivary glands (g)				
Both parotid	23·5	15·7–30·6	65·3	50·4–95·4
Both submaxillary	18·2	11·8–24·2	64·0	47·6–84·9
Both inferior molar	5·9	4·5– 8·7	13·5	8·5–19·5
Both ventral sublingual	1·3	0·8– 1·6	11·3	8·5–14·7
Both buccal	6·0	4·2– 8·8	13·1	8·5–22·0
Palatine	8·8	5·9–12·1	17·2	13·3–19·1
Pharyngeal	5·9	4·1– 7·1	9·1	6·4–12·9
Labial	10·9	4·5–16·4	8·9	7·0–11·6

In Table 2 the weights of the parotid, submaxillary and inferior molar glands are compared in a group of twenty-four sheep. Judging by the correlation coefficients, body weight was not a particularly good measure of the weight of the salivary glands, which varied greatly from one sheep to another. Body weight was a still poorer measure of the weight of single salivary glands, for the weights of the left and right glands of individual animals sometimes differed considerably. For example, in eleven of the

twenty-four sheep in Table 2 the weights of the two parotid glands differed by less than 10%, in nine they differed by 10–20%, in two by 25 and 28%, and in two by 50 and 51%.

TABLE 2. The weights of salivary glands in twenty-four adult sheep and their correlation with body weight

	Mean	Range	Correlation coefficient (r), salivary gland wt. with body wt.
Body weight (kg)	41	31–52	
Salivary glands (g)			
Both parotid	21·4	14·9–30·6	+0·61*
Both submaxillary	18·1	11·8–29·7	+0·51*
Both inferior molar	5·0	2·3– 8·7	+0·40†

* Significant at $P = 0{\cdot}01$; † significant at $P = 0{\cdot}05$.

Histological appearance. In adult ruminants the parotid, submaxillary and sublingual glands have been characterized as serous, mixed and predominantly mucous glands respectively (Bock & Trautmann, 1914; Ziegler, 1927, 1929). In young ruminants, however, the parotid gland frequently contains groups of mucous cells (Bock & Trautmann, 1914) and the sublingual gland groups of serous cells (Ziegler, 1929). No report has been found concerning the histological appearance of the small glands of the mouth and so the distribution of serous and mucous cells in these glands was compared with their distribution in the major glands. Material was fixed in 10% formol-saline and embedded in wax. Sections were stained with haematoxylin and eosin, with haematoxylin and periodic acid-Schiff reagent (P.A.S.), or with haematoxylin and mucicarmine. The glands could be divided into three groups:

Group 1, Serous: parotid and inferior molar glands (Pl. 1, figs. 1–4). The alveoli of these glands were small and the cytoplasm of the secretory cells did not stain with P.A.S. or mucicarmine. There were many intralobular ducts lined with cubical epithelium and a few smaller ducts, intercalary ducts, lined by flat epithelial cells. Although there were no mucous cells in the gland, goblet cells were present in the primary duct, sometimes in sufficient numbers to form an almost continuous mucous epithelium, and a few were found in the larger interlobular ducts. The parotid and inferior molar glands were histologically identical.

Group 2, Mucous: palatine, pharyngeal and buccal glands (Pl. 1, figs. 5 and 6). The alveoli were larger than those of the serous glands. The secretory cells were large and often distended with granules that stained with P.A.S. and mucicarmine. No goblet cells were found in the ducts. The intralobular ducts were lined with flat epithelial cells.

Group 3, Mixed: submaxillary, sublingual and labial glands (Pl. 2,

figs. 1–6). The alveoli were lined with large cells that stained strongly with P.A.S. and contained granules that stained with mucicarmine. In the submaxillary gland the alveoli were capped by demilunes of smaller serous cells that did not stain with P.A.S. or mucicarmine and roughly equal numbers of mucous and serous cells were present. In the sublingual and labial glands there were fewer serous cells and only occasionally were they grouped to form small demilunes. In all the glands many intralobular ducts lined with columnar epithelium were found. There were no goblet cells in the ducts.

Rates of secretion of saliva

Parotid and submaxillary saliva. Saliva was collected for 24 hr from one parotid and one submaxillary gland in eleven conscious stall-fed sheep. The volumes of saliva secreted are given in Table 3. The volume of parotid saliva secreted daily varied greatly between sheep. This variation did not correlate with food intake nor with gland weight ($r = 0{\cdot}28$, not significant) but possibly with body weight ($r = 0{\cdot}57$, $P = 0{\cdot}05$). In a second series of

TABLE 3. The volumes of parotid and submaxillary saliva secreted during 24 hr by eleven conscious sheep

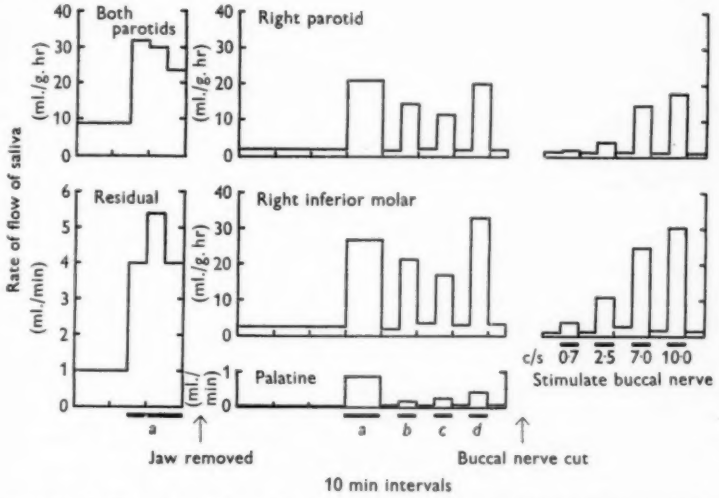
Breed	Body weight (kg)	Food intake (g)		Parotid weight (g)*	Saliva volume (ml./24 hr)	
		Hay	Meals		One parotid	One submaxillary
Shetland	33	550		7·3	1260	375
	35	450		—	2140	310
Blackface	27	500		9·8	2510	310
	33	550		7·3	1760	350
	35	600		7·3	2350	225
	43	750	225	—	2040	340
	44	500		—	1940	260
	45	850		—	3030	340
	46	800		9·5	2310	185
	46	900		—	3110	355
	49	600		11·7	3720	340
Mean	40	650	225	8·8	2380	310

* Some of the sheep were still alive at the time of writing.

experiments on six Blackface sheep weighing 41–45 kg, parotid saliva alone was collected and 1370–4230 ml./24 hr was obtained. When parotid saliva was collected from one sheep on successive days, however, the volume remained within fairly narrow limits (Text-fig. 4).

The submaxillary gland secreted only about one-eighth as much saliva as the parotid gland. Most of this saliva was secreted during periods of feeding. For example, in five sheep 56–78 % of the submaxillary saliva collected during 24 hr was secreted during the 3·0–5·9 hr of feeding time.

Residual and sublingual saliva. Kay & Phillipson (1959) found that after cannulation of the parotid and submaxillary glands the flow of residual saliva from the mouth under conditions of alternating rest and reflex stimulation was similar in volume to that of the parotid saliva. The ventral sublingual glands, when cannulated, were found to be secreting only slowly. The inferior molar glands and the small mucous glands have therefore been studied to see if their secretion would account for the residual saliva.



Text-fig. 3. The rate of flow of various salivary secretions in an anaesthetized sheep. On the left is shown the flow of saliva from both parotid glands and of the residual saliva dripping from the mouth; these flows were increased by distension of the oesophagus (*a*). The jaw was then removed and saliva was collected from the right parotid gland, a lobe of the right inferior molar gland and from the palate. These flows were accelerated by distension of the oesophagus, (*a*), stretch of the reticulo-omasal orifice (*b*), gentle stroking of the reticulo-rumen fold and of the floor of the anterior sac and the anterior pillar of the rumen (*c*), and stretch of the reticulo-rumen fold (*d*). Finally, stimulation of the peripheral end of the buccal nerve at the frequencies indicated (c/s) caused similar increases in the flows of parotid and of inferior molar saliva.

Text-fig. 3 gives the result of an experiment on a sheep anaesthetized with chloralose in which the parotid and submaxillary glands were cannulated and the ducts of the sublingual glands were cut to prevent the entry of sublingual saliva into the mouth. The rates of secretion of parotid and residual saliva were measured under conditions of rest and when reflexly stimulated by distension of the thoracic oesophagus with a condom. Rubbing a brush between the molar teeth and tongue, another effective

stimulus to parotid secretion (Kay, 1958), was also found to increase the flow of residual saliva.

The rami of the mandible were then divided and the lower jaw was removed, leaving the cheeks and their glands attached to the upper jaw. The sheep was placed on its back and saliva was collected from a posterior lobe of one inferior molar gland, after cannulation of the duct, and from the palate. Most of the latter was secreted by the palatine glands but a small contribution came from the more anterior pharyngeal glands and the glands at the root of the tongue.

The parotid and inferior molar glands secreted continuously and responded equally to a variety of stimuli applied to the oesophagus and reticulo-rumen. Small beads of saliva appeared over the ducts of the palatine glands during periods of rest, but the volume of this continuous secretion was small compared to that of the parotid and inferior molar glands. The palatine glands responded to all the stimuli that increased parotid and inferior molar secretion. The buccal glands and the pharyngeal glands that were visible were seen to respond similarly, but the labial glands were not appreciably affected.

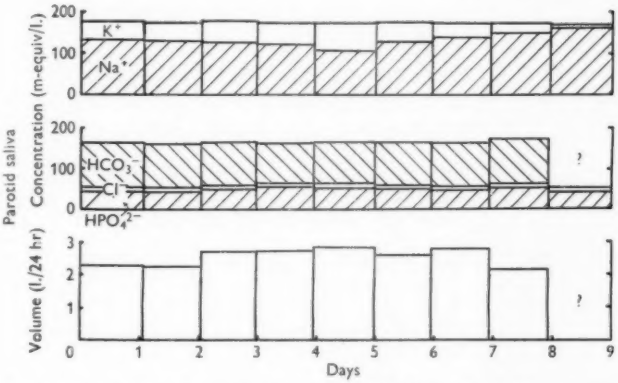
In this sheep the saliva collected from the palate represented the secretion of about half of the total mass of the small mucous glands of the mouth, and so the rate of mucous secretion during oesophageal distension would probably have totalled 1–2 ml./min. The weight of both inferior molar glands was 4·5 g, so that the rate of inferior molar secretion during oesophageal distension, 27–33 ml./g. hr, corresponded to a total flow of 2·0–2·5 ml./min. The combined flow of mucus and inferior molar saliva thus accounted for almost all the residual saliva, which flowed at a mean rate of 4·5 ml./min during oesophageal distension. The parotid and inferior molar glands continued to secrete at a slow rate after section of the ipsilateral buccal nerve, but failed to respond to reflex stimulation. Electrical stimulation of the peripheral end of the buccal nerve caused both glands to secrete rapidly and also caused the buccal glands and some of the labial glands to secrete; the palatine glands were not affected.

Similar results were obtained in four other experiments. The rate of secretion of the inferior molar gland, expressed as ml./g.hr, was generally similar to or slightly greater than that of the parotid gland, whether the glands were resting, denervated or stimulated reflexly or directly. The factors controlling secretion by the inferior molar gland therefore appear to be identical with those controlling parotid secretion.

The rates of secretion of single ventral sublingual glands are given in Table 7. The samples were collected under conditions of alternating rest and reflex stimulation of the parotid from the oesophagus and reticulo-rumen, but no stimuli were applied to the mouth.

Composition of saliva

Parotid saliva. Observations made in the present experiments on changes in the composition of parotid saliva of sheep caused by altering the sodium intake or the rate of parotid secretion wholly support the conclusions of Denton (1956) and of Coats & Wright (1957). For example, parotid saliva was collected continuously for 8 days from an adult sheep, Sheep 5, that had been trained to drink saliva from a bottle. During the first 4 days the collected saliva was returned in this manner, and the saliva K^+ concentration was found to be rising progressively. A sodium-rich artificial saliva (Na^+, 185; HCO_3^-, 117; HPO_4^{2-}, 52; Cl^-, 16 m-equiv/l.) was then returned in place of the collected saliva and the saliva K^+ concentration returned to levels characteristic of the sodium-sufficient sheep (Text-fig. 4).



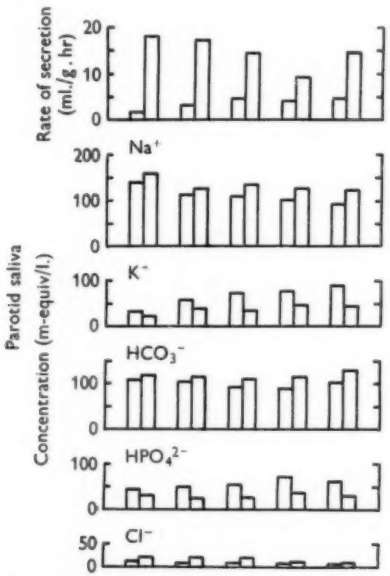
Text-fig. 4. The volume and composition of parotid saliva collected from a conscious sheep. The collected saliva was returned for the first 4½ days of the experiment. For the second 4½ days a sodium-rich artificial saliva, containing (mM) sodium 180, bicarbonate 120, phosphate 25 and chloride 10, was returned. Intermittent blockage of the cannula occurred on the last day.

Ruminant parotid secretion is greatly increased during feeding. Pairs of saliva samples were taken at different stages of the sodium depletion-repletion cycle described above, the first while the sheep was idling and the second 5–10 min later when the sheep was eating hay. The rate of secretion of the second sample was 2–10 times faster than that of the first sample and the effect of this increased flow on the composition of the saliva is shown in Text-fig. 5. The greatest differences in composition between the samples occurred when the concentrations of Na^+ and HCO_3^- before feeding were depressed and those of K^+ and HPO_4^{2-} were elevated.

The concentration of Na^+ and K^+ in parotid saliva showed a strong

inverse correlation and the concentrations of HCO_3^- and HPO_4^{2-} showed a less marked inverse correlation. In Sheep 3 and 5, the two sheep that were studied most intensively, the correlation coefficients for Na^+ and K^+ were $-0{\cdot}96$ and $-0{\cdot}97$, and those for HCO_3^- and HPO_4^{2-} were $-0{\cdot}70$ and $-0{\cdot}87$, respectively.

A related observation was that the total concentrations of measured cations and anions varied only within narrow limits and that the variations that did occur were not related to the rate of secretion (Table 4). It is not known why the total of cations was greater than the total of anions but this discrepancy appeared in all sheep that were examined.



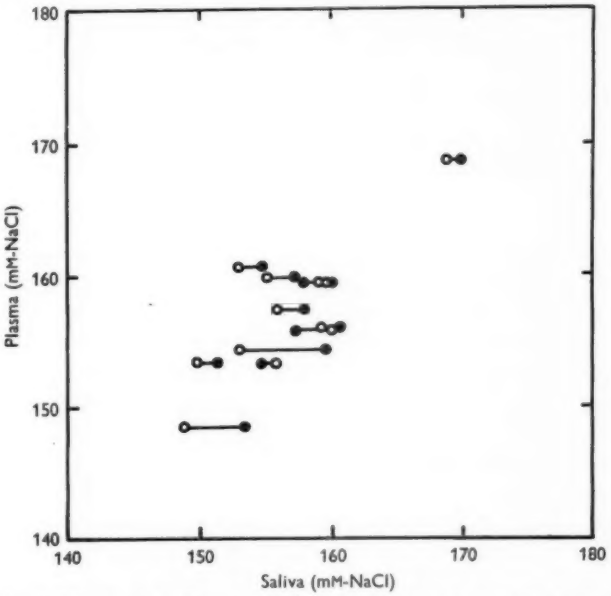
Text-fig. 5. The influence of the rate of secretion of parotid saliva on its composition. Five pairs of saliva samples were collected from a conscious sheep, the first of each pair at a slow rate of secretion immediately before feeding, and the second at a rapid rate of secretion during feeding.

TABLE 4. Total concentrations of cations ($Na^+ + K^+$) and of anions ($HCO_3^- + HPO_4^{2-} + Cl^-$) in parotid saliva at slow (< 4 ml./g.hr) and fast (> 4 ml./g.hr) rates of secretion in two conscious sheep

	No. of samples	Mean rate of secretion (ml./g.hr)	Cations (m-equiv/l., mean ± S.E.)	Anions (m-equiv/l., mean ± S.E.)
Sheep 3	32	2·4	169 ± 1·1	159 ± 1·0
	11	6·9	169 ± 1·7	164 ± 1·5
Sheep 5	20	2·8	171 ± 1·4	164 ± 1·5
	27	8·4	173 ± 1·0	164 ± 1·0

These results suggested that the parotid saliva of sheep has an almost constant tonicity. In order to find whether the parotid saliva was isotonic with plasma, measurements were made of the freezing points of plasma and of saliva samples collected just before and during feeding. There was no significant difference in freezing point between plasma and either saliva sample. The results for each of the twelve sheep studied are illustrated in Text-fig. 6 and further relevant data are given in Table 5.

Submaxillary saliva. Table 6 compares the compositions of the submaxillary and parotid salivas that were collected from the eleven conscious



Text-fig. 6. The tonicity of jugular plasma and of pairs of parotid saliva samples collected from 12 conscious sheep. One saliva sample (○) was collected at a slow rate of secretion immediately before feeding and the second (●) at a rapid rate of secretion during feeding. The freezing points were measured with a Beckmann thermometer and converted to the equivalent concentration of NaCl according to the relation shown in Fig. 1.

TABLE 5. Freezing points of parotid saliva and plasma; mean values ± S.D. for samples taken from twelve conscious sheep

	Rate of secretion (ml./min)	Freezing point (mM-NaCl)	Saliva f.p. / plasma f.p.	$Na^+ + K^+$ (m-equiv/l.)
'Idling' saliva	0·9	157 ± 4·9	0·996 ± 0·017	190 ± 14
'Feeding' saliva	7·8	158 ± 4·5	1·003 ± 0·022	183 ± 6
Plasma	—	157 ± 4·6	—	158 ± 6

sheep in which both glands were cannulated. The submaxillary saliva differed from the parotid saliva in that it had much lower concentrations of Na^+, HCO_3^- and HPO_4^{2-}; the concentrations of K^+ and Cl^- were about the same in each secretion. As a result, it had a much smaller buffering power. In one sheep, for example, it required only 0·3 ml. of N-HCl to titrate 20 ml. of submaxillary saliva to pH 5, whereas 20 ml. of parotid saliva required 2·8 ml. of the acid. The submaxillary saliva was mucous and slightly cloudy while the parotid saliva was fluid and clear.

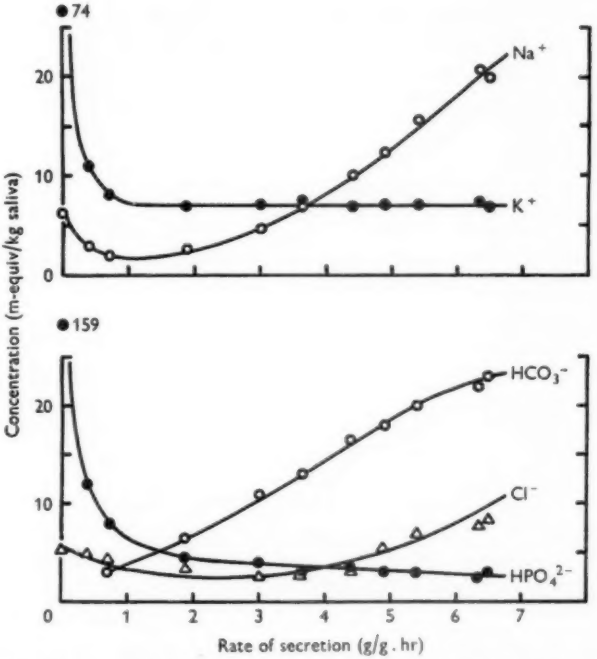
TABLE 6. Composition of 24 hr collections of parotid and submaxillary saliva from eleven conscious sheep

	m-equiv/l.				
	Na^+	K^+	HCO_3^-	HPO_4^{2-}	Cl^-
Parotid					
Mean	170	13	112	48	11
Range	147–185	6–31	103–125	25–64	9–16
Submaxillary					
Mean	9	16	9	5	11
Range	4–16	10–25	5–14	2–10	7–15

Five experiments on conscious sheep indicated that sodium depletion influences not only the composition of parotid saliva but also that of submaxillary saliva. One parotid and one submaxillary gland were cannulated and the secretions were collected for two 24-hr periods, the first while a sodium-rich artificial saliva was being introduced into the rumen at a rate of 2 l./day and the second 24 hr after starting to introduce a potassium-rich artificial saliva at the same rate. The artificial salivas contained 180 mM sodium or potassium, 120 mM bicarbonate, 25 mM phosphate and 10 mM chloride. The $Na^+:K^+$ ratio of the parotid saliva during the first collection period was 20·5 (range 9·7–30·3) and that of the submaxillary saliva was 0·71 (range 0·50–0·90). In the second collection period, during sodium depletion, these ratios were reduced to 4·6 (range 2·3–9·0) for the parotid saliva and to 0·25 (range 0·15–0·42) for the submaxillary.

Three experiments were performed under anaesthesia to study the influence of the rate of secretion on the composition of submaxillary saliva. The submaxillary duct was cannulated and the rate of secretion was controlled by electrical stimulation at various frequencies of the peripheral end of the divided submaxillary nerve. Text-fig. 7 shows the changes in composition that occurred. The sample recorded at zero flow consisted of the 2nd–6th drops of saliva to be collected during the first period of stimulation. This was the volume that could be massaged from the gland and so its composition probably approximated to that of the saliva that was lying stagnant in the ducts before stimulation. Considerable concentration of K^+, HPO_4^{2-} and, in some preparations, of Na^+ occurred at zero and very

low rates of flow. As the rate of flow was increased to 1 g/g. hr the concentrations of Na^+, K^+, HPO_4^{2-} and Cl^- fell. At faster flows the concentrations of Na^+, HCO_3^- and Cl^- increased, K^+ remained constant and HPO_4^{2-} continued to fall. The viscosity of the saliva increased with the rate of flow.



Text-fig. 7. The composition of submaxillary saliva at different rates of secretion in an anaesthetized sheep. The flow of saliva was controlled by intermittent stimulation (5 sec on, 5 sec off) of the peripheral end of the submaxillary nerve with a square wave pulse of variable frequency.

The composition of submaxillary saliva collected from anaesthetized sheep and calves during the experiments reported by Kay & Phillipson (1959) is shown in Table 7. In general the composition of the sheep samples was intermediate between the zero flow and the most slowly secreted samples shown in Text-fig. 7, as would be expected from their slow rates of secretion. The calf samples differed in not having high HPO_4^{2-} concentrations. Although the submaxillary samples collected from the conscious sheep had a rate of flow, 0·05–0·10 ml./min, which was similar to that of the samples collected from anaesthetized sheep, their composition differed from the latter in that the concentrations of Na^+, K^+ and HPO_4^{2-}

were lower. This may have been due to the fact that the bulk of the 24-hr sample was secreted at a rapid rate during periods of feeding.

Sublingual saliva. The composition of sublingual saliva taken from six sheep under chloralose anaesthesia is given in Table 7. The saliva was clear and very mucous.

TABLE 7. The composition of submaxillary, sublingual and labial saliva collected from anaesthetized sheep and calves

	Rate of secretion		m-equiv/l.				
	ml./min	ml./g.hr	Na^+	K^+	HCO_3^-	HPO_4^{2-}	Cl^-
Submaxillary saliva, 7 sheep:							
Mean	0·10	0·42	15	26	6	54	6
Range	0·004–0·35	0·01–1·78	3–66	15–51	1–9	14–175	2–9
4 calves:							
Mean	0·05	0·05	18	33	6	1·5	9
Range	0·01–0·08	0·01–0·08	11, 24*	24, 41*	5–8	0·4–4·0	6–15
Sublingual saliva, 6 sheep:							
Mean	0·009	0·79	30	11	12	0·9	28
Range	0·002–0·013	0·24–1·60	16–47	6–25	8–18†	0·3–2·0‡	16–40
Labial saliva, 3 sheep:							
Mean	—	—	39	6	3	5	34*
Range	—	—	29–47	3–9	2, 4†	2–10	

* 2 samples. † 5 samples. ‡ 4 samples.

Residual saliva. Samples of parotid and residual saliva were collected from seven anaesthetized sheep and four anaesthetized calves. The submaxillary ducts were separately cannulated and the sublingual ducts were cut. The inorganic composition of the residual saliva in animals that were not sodium-depleted was similar to that of the parotid saliva (Tables 8 and 9). At slow rates of secretion the residual saliva was fluid enough to form drops, but when the flow was stimulated reflexly by distension of the thoracic oesophagus the saliva consisted of a mucous component that formed thick strands hanging from the mouth, and a fluid component that flowed over the surface of these strands.

Samples of palatine and inferior molar saliva were collected from sheep after removal of the lower jaw. The palatine saliva was clear and very mucous and evidently contributed to the mucous component of the residual saliva, while the inferior molar saliva supplied the fluid component. Both palatine and inferior molar salivas were generally similar to parotid saliva in their inorganic composition, although the palatine saliva had somewhat lower concentrations of HPO_4^{2-} and HCO_3^- and a correspondingly higher concentration of Cl^- (Table 8).

In three experiments labial saliva was aspirated from between the lips and dental pad after a period of stimulation of the buccal nerve. It was a

clear and mucous saliva, similar to palatine saliva in appearance, although its inorganic composition, given in Table 7, more closely resembled that of sublingual saliva.

Similar experiments were conducted on two sodium-depleted sheep to determine which of the glands contributing to the residual saliva were affected by this condition. Sodium depletion was caused by loss of rumen fluid through leaky rumen fistulae. The results for one sheep are given in Table 8 and the second sheep behaved similarly. In both parotid and inferior molar saliva the concentration of Na^+ was reduced and that of K^+ increased, but the palatine saliva remained similar in composition to the palatine saliva of normal sheep. As would be expected, the residual saliva was intermediate in composition between inferior molar and palatine saliva.

TABLE 8. The composition of various salivary secretions from a normal and a sodium-depleted sheep under anaesthesia

Saliva	Rate of secretion	m-equiv/l.				
		Na^+	K^+	HCO_3^-	HPO_4^{2-}	Cl^-
Normal sheep						
Parotid (resting)	3·0 g/g.hr	189	5	91	79	9
Parotid (reflex)	7·8 g/g.hr	182	5	99	71	16
Residual (resting)	0·7 ml./min	189	6	92	70	13
Residual (reflex)	3·2 ml./min	174	6	107	44	14
Inferior molar (resting)	3·3 g/g.hr	175	7	110	51	12
Inferior molar (reflex)	7·8 g/g.hr	175	10	97	44	7
Palatine (reflex)	0·5 ml./min	179	4	109	25	25
Sodium-depleted sheep						
Parotid (resting)	1·0 g/g.hr	20	111	71	60	4
Parotid (reflex)	1·4 g/g.hr	47	102	75	61	4
Residual (resting)	0·3 ml./min	92	56	69	33	33
Residual (reflex)	0·8 ml./min	130	44	89	29	31
Inferior molar (reflex)	0·6 g/g.hr	42	99	38	87	11
Palatine (reflex)	0·1 ml./min	171	13	56	25	45

The composition of the saliva collected from the epithelium of the cheeks, a mixture of the secretions of the inferior molar and buccal glands, was also intermediate between inferior molar and palatine saliva, indicating that the composition of buccal saliva is similar to that of palatine saliva.

In calves, ducts of the inferior molar gland were isolated and cannulated after making a skin incision over the lateral border of the mandible and reflecting the gland dorsally. The composition of parotid, residual and inferior molar saliva collected from two calves is given in Table 9. The parotid and inferior molar secretions were similar to each other in each calf, but differed between calves.

TABLE 9. The composition of parotid, inferior molar and residual saliva in two anaesthetized calves

	m-equiv/l.				
	Na^+	K^+	HCO_3^-	HPO_4^{3-}	Cl^-
Calf 3					
Parotid	163	14	88	47	16
Inferior molar	156	18	77	54	12
Residual	162	12	66	34	55
Calf 4					
Parotid	168	6	94	17	34
Inferior molar	151	6	95	18	21
Residual	165	8	75	20	50

DISCUSSION

The results of this investigation are summarized in Table 10. The daily volume of inferior molar saliva is calculated from that of parotid saliva on the assumption that the rate of secretion in ml./g.hr is the same for both glands and that the weight of the inferior molars is 25 % of that of the parotids. The volume of saliva secreted by the small mucous glands is estimated by assuming that the residual saliva is about equal in volume to the parotid saliva, as has been found by Scheunert & Krzywanek (1929), Phillipson & Reid (1958) and Kay & Phillipson (1959), and that the small mucous glands and the inferior molar glands together secrete all the residual saliva. Ilgner (1921) reported the ratio of inferior molar to parotid weights to be 1:2·9 in sheep. Scheunert & Krzywanek (1929) consequently suggested that the inferior molar glands secreted the greater part of the saliva they collected from an oesophageal fistula after cannulation of both parotid ducts; this appears to underestimate the contribution of the small mucous glands.

Since the volume of the residual saliva is approximately equal to that of the parotid saliva it is likely that the total volume of saliva secreted by sheep will be about twice that of the parotid saliva with an addition of less than 1 l. of submaxillary and sublingual saliva, i.e. about 6–16 l./24 hr. On the other hand Somers (1957) estimated the total salivary secretion of sheep to be only 3–6 l./24 hr, but may not have been aware of the importance of the residual saliva.

Like parotid saliva, the residual saliva seems to be as important in the control of the consistency and pH of the rumen contents as in assisting mastication and deglutition. In addition it may be a special function of the palatine and pharyngeal glands to coat boli of food with mucus as they are swallowed. This mucous coat is a notable feature of boli collected from the cardia. The secretions of the submaxillary and sublingual glands, on the other hand, are much smaller in volume and only weakly buffered,

TABLE 10. Summary of characteristics of the salivary glands of sheep

Glands	Mean weight (g) 6 sheep	Weight relative to parotids (%)	Cell type	Factors governing rate of flow	Estimated saliva volume (l./24 hr)	Saliva type	Effect of sodium depletion
Both parotid	23·5	100	Serous	Continuous flow when denervated. Respond to stimulation of mouth, oesophagus and reticulo-rumen	3–8	Fluid and isotonic. Strongly buffered with HCO_3^- and HPO_4^{2-}	Replacement of salivary Na^+ with K^+
Both inferior molar	5·9	25	Serous	Continuous flow when denervated. Respond to stimulation of mouth, oesophagus and reticulo-rumen	0·7–2·0	Fluid and isotonic or nearly so. Strongly buffered with HCO_3^- and HPO_4^{2-}	Replacement of salivary Na^+ with K^+
Palatine, buccal and pharyngeal	20·7	88	Mucous	Very slow continuous flow when not stimulated. Respond to stimulation of mouth, oesophagus and reticulo-rumen	2–6	Very mucous and isotonic or nearly so. Strongly buffered with HCO_3^- and HPO_4^{2-}	Little or no effect
Both submaxillary	18·2	77	Mixed	No flow when denervated. Strongly stimulated by feeding. Little or no response to stimulation of oesophagus or reticulo-rumen	0·4–0·8	Variably mucous and hypotonic. Weakly buffered	Replacement of salivary Na^+ with K^+
Both sublingual	1·3	6	Mixed	Continuous flow when not stimulated. Moderately stimulated from oesophagus; other reflexes not studied	0·1 (?)	Very mucous and hypotonic. Weakly buffered	?
Labial	10·9	46	Mixed	Little or no flow when not stimulated. Little or no response to stimulation of oesophagus and reticulo-rumen; other reflexes not studied	?	Very mucous and hypotonic. Weakly buffered	?

and so will contribute little to the control of acidity in the rumen. Colin (1886) and Scheunert & Trautmann (1921) have shown that these glands are active during mastication of food although not during rumination, and their chief importance may be to moisten and lubricate food during mastication. The little that is known of the labial glands suggests that they are similar to the submaxillary and sublingual glands in performance and function.

Parotid glands. The weights of both parotid glands in sheep listed in this paper have a range, 14·9–30·6 g, similar to that reported by Denton (1957*c*), 13·8–43·5 g, for Merino, Merino Cross and Suffolk sheep.

The daily volumes of parotid saliva given in Table 3 are similar to those recorded for stall-fed sheep by earlier workers. The rates of secretion and the techniques employed are summarized in Table 11.

TABLE 11. Published data on the output of parotid saliva in stall-fed sheep

Output/24 hr for one parotid	Technique	Reference
930–1840 ml.	Saliva collected from one cannulated parotid duct and partially returned	McDougall (1948)
1·0–4·0 l.	Saliva collected from one transplanted parotid papilla and replaced by a $NaHCO_3$ supplement	Denton (1957*b*)
1992–2775 ml.	Saliva flow recorded alternately from each parotid with continuous reinfusion of saliva by an orally-directed parotid duct cannula	Somers (1957)
3·91–4·24 l.*	Saliva flow recorded from both parotids with continuous reinfusion of saliva by orally-directed parotid duct cannulae	Stewart & Dougherty (1957)
1260–4230 ml.	Saliva collected from one cannulated parotid papilla and returned into the rumen intermittently	This paper

* Output for both parotids.

In cattle the volume of saliva secreted whilst feeding depends on the amount and coarseness of the food (Colin, 1886; Denton, 1957*b*; Balch, 1958). It is therefore surprising that the variable volume of parotid saliva collected from different sheep did not correlate with the different amounts of food ingested. The analysis of this variation in terms of parotid gland weight and body weight yielded little information but this may have been due partly to the small number of animals studied. Another possibility is that salivary secretion was inhibited to some extent as a result of restraining the sheep in recording stands during the period of collection and that this inhibition was more marked in some sheep than in others. Denton (1957*a*) showed that parotid secretion was wholly inhibited in untrained sheep when they were forcibly laid on their sides.

The changes in the composition of parotid saliva caused by sodium depletion and by alteration of the rate of secretion further confirm the careful work of Denton (1956) and of Coats & Wright (1957). Denton (1957*b*) found that the total electrolyte concentration of parotid saliva was considerably greater than that of plasma and thus regarded the saliva as being hypertonic to plasma. Measurements of the osmotic pressure of the fluids by freezing-point determinations show that in fact the saliva is isotonic with plasma over a wide range of rates of saliva secretion and plasma osmotic pressure. Differences in the concentrations of unmeasured electrolytes and organic solutes in the saliva and plasma presumably balance the difference in the measured electrolyte concentrations. The relatively constant tonicity of the saliva explains the strong negative correlations found between the concentrations of Na^+ and K^+, the principal cations, and between HCO_3^- and HPO_4^{2-}, the principal anions. Even if Na^+ and K^+ were the only cations present, however, an exact inverse relationship would not be expected between their concentrations, for in a solution of constant tonicity the total concentration of Na^+ and K^+ will vary directly with the concentration of divalent anions, principally HPO_4^{2-}.

Unlike the salivary glands of non-ruminant animals, which secrete a hypotonic saliva, the ruminant parotid gland does not appear to perform any osmotic work. For this reason it is possible that the alveoli or ducts are freely permeable to water. Osmotic work accounts for a large part of the energy expenditure of the dog parotid gland (Burgen, 1955), and to be spared this work may enable the ruminant parotid to achieve the very high rates of secretion, 7·4–32·5 ml./g.hr, of which it is capable (Kay & Phillipson, 1959). The mean plasma osmotic pressure, corresponding to 157 mM-NaCl (0·921 g NaCl/100 g water), is somewhat lower than that obtained by Aldred (1940), 0·978 g NaCl/100 g water, by determinations of the vapour pressure of sheep blood.

Small glands of the mouth. Zimmermann (1927) gives a detailed account of the appearance and distribution of the human salivary glands. Only the inferior molar gland of the sheep has no counterpart in man. The inferior molar gland resembles the parotid gland in its histological appearance, its innervation through the buccal nerve, its rate of secretion and reflex control and in the composition of its saliva. Goblet cells are found in the ducts of the inferior molar gland as they are in the parotid duct (Bock & Trautmann, 1914).

The small mucous glands of the sheep are similar to the small human salivary glands in their distribution but differ to some extent in their relative sizes and in histological appearance. The ruminant is not exceptional in secreting large volumes of residual saliva. Montgomery & Stuart (1936) showed that the labial, molar, palatine, and other mucous glands of

dogs readily respond to chemical and mechanical stimulation of the mouth and to conditioned stimuli. One conscious dog with bilaterally cannulated parotid and submaxillary glands secreted up to 5·85 g of mucus from an oesophageal fistula and up to 19·38 g of parotid and submaxillary saliva in 5 min. The palatine glands of cats, rabbits, guinea-pigs and rats also secrete considerable quantities of mucus (Cohen & Myant, 1959).

Although the microscopic appearance of the palatine glands of the sheep is so different from that of the parotid gland the inorganic composition of palatine saliva differs from normal parotid saliva only in containing rather more Cl^- and proportionately less HPO_4^{2-} and HCO_3^-. In sodium-depleted sheep this similarity disappeared, for the Na^+ of palatine saliva remained at a high level although that of the parotid saliva had largely been replaced by K^+. The buccal glands behaved in the same way, and so the small mucous glands of the mouth will continue to supply substantial quantities of Na^+ to the rumen despite dietary sodium deficiency.

The approximately isotonic nature of palatine saliva may be related to the absence of intralobular ducts lined with columnar epithelium, for Bro-Rasmussen, Killman & Thaysen (1956) and Burgen & Seeman (1958) have presented evidence suggesting that these ducts are responsible for the hypotonicity of human and dog saliva. In sheep also a hypotonic saliva is secreted by the submaxillary, sublingual and labial glands, which contain such ducts.

Submaxillary glands. These are the only mixed mucous and serous glands of any size in the sheep. The composition of sheep submaxillary saliva and the manner in which the composition changes with the rate of secretion are similar to those found for the submaxillary glands of the dog (Gregersen & Ingalls, 1931) and cat (Langstroth, McRae & Stavraky, 1938) while the changes in the Na^+:K^+ ratio during sodium depletion compare with variations in the Na^+:K^+ ratio of human mixed saliva that are found under conditions of adrenal cortical overactivity and insufficiency (Frawley & Thorn, 1951). The volume of submaxillary saliva secreted in 24 hr was only about one eighth of that secreted by the parotid glands and, as was found by Colin (1886) and by Scheunert & Trautmann (1921), most of it was secreted during feeding periods.

The composition of submaxillary saliva collected from anaesthetized calves is generally similar to that reported by Mangan (1959). As in the case of sheep, the submaxillary saliva secreted by conscious calves may be rather different in composition.

Sublingual glands. These small glands probably secrete little saliva. The fastest rate of secretion recorded by Kay & Phillipson (1959) was 0·033 ml./min for a single sublingual gland, equivalent to 95 ml./24 hr for both glands. This flow occurred during distension of the oesophagus, a stimulus

unlikely to increase sublingual secretion as much as feeding. However, feeding only occupies a small fraction of the day in stall-fed sheep and so the daily volume of sublingual saliva is unlikely to exceed 100 ml. (Table 10). In cattle, where the sublingual gland is relatively larger than in sheep, Colin (1886) reports that 18–20 g of sublingual saliva is secreted per hour. This corresponds to 864–960 g/24 hr for both sublingual glands, a very small part of the total salivary secretion of 56 kg/24 hr.

SUMMARY

1. The weights of the salivary glands of sheep and calves were measured. The small serous and mucous glands of the mouth constitute a considerable part of the total salivary gland mass. The distribution and histological appearance of the glands are described.

2. In conscious sheep 1260–4230 ml. of parotid saliva and 185–375 ml. of submaxillary saliva were collected from single glands per day. The compositions of these secretions are compared.

3. The changes in the composition of parotid saliva caused by sodium depletion and by changing the rate of secretion that have been reported by previous workers are confirmed.

4. Measurements of the freezing point of parotid saliva and plasma indicate that the two fluids are isotonic.

5. The serous inferior molar glands appear to be identical with the parotid glands in their histological appearance and in their rate of secretion. Inferior molar saliva is similar to parotid saliva in composition.

6. The submaxillary, sublingual and labial salivas contain much lower concentrations of Na^+, HCO_3^- and $H_2PO_4^{2-}$ than parotid saliva. The Na^+:K^+ ratio of submaxillary saliva is depressed by sodium depletion and the composition of the saliva is considerably influenced by the rate of secretion.

7. The mucous secretions of the palatine, buccal and pharyngeal glands are increased by stimuli which increase parotid and inferior molar secretion. The inorganic composition of palatine saliva is similar to that of parotid saliva except that there is little or no fall in the Na^+ concentration during sodium depletion.

8. The daily volumes of saliva secreted by different glands are estimated.

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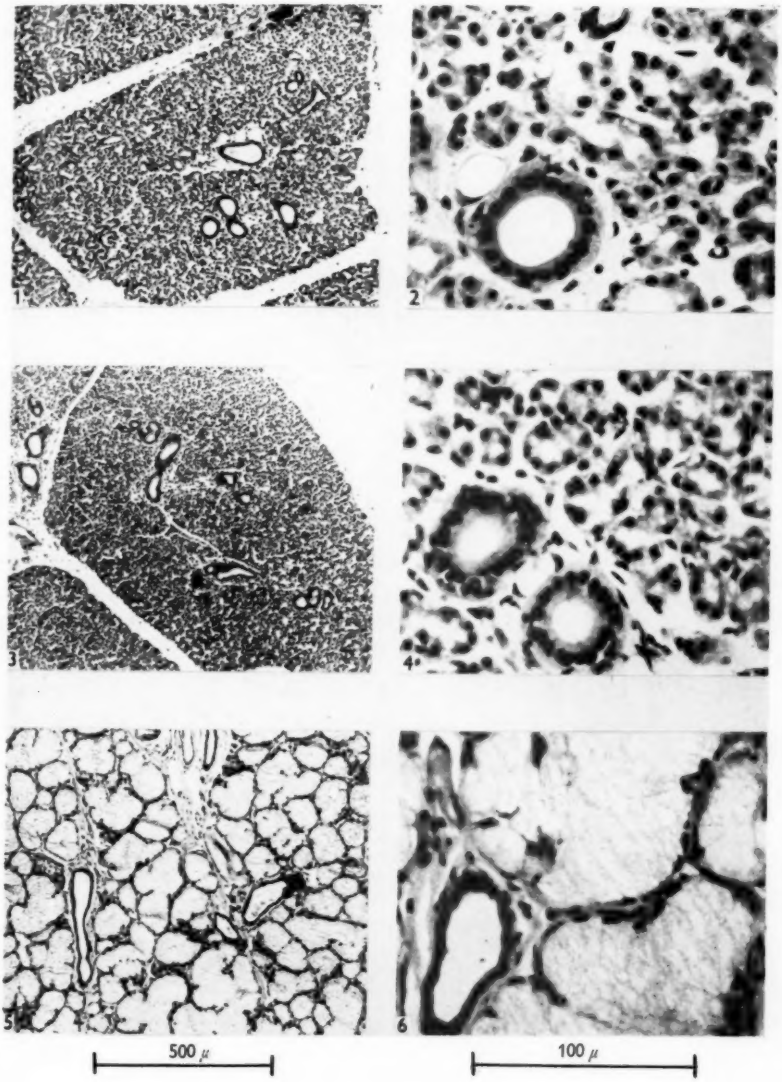

R. N. B. KAY

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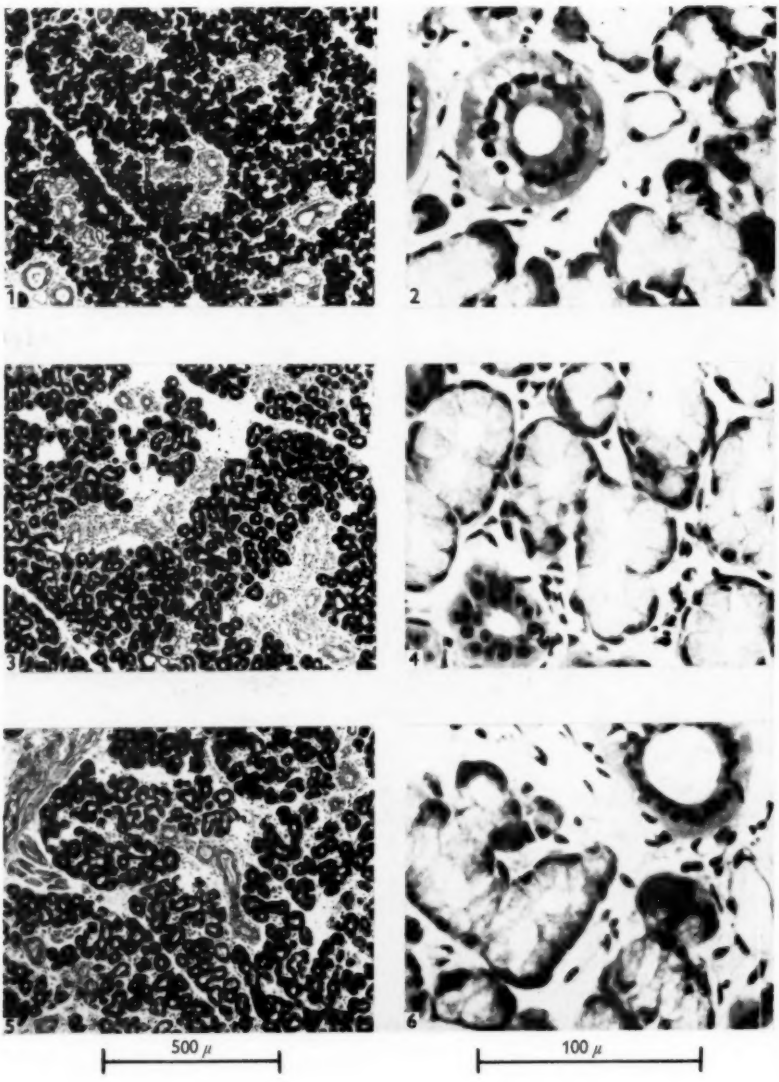
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EXPLANATION OF PLATES

5 μ sections of salivary glands fixed by perfusion of the head with 0·9 % NaCl solution followed by 10 % formol-saline.

Plate 1

Figs. 1 and 2, parotid gland; figs. 3 and 4, inferior molar gland; haematoxylin-eosin. Closely packed serous cells and occasional ducts lined with cubical epithelium are present in both glands. Figs. 5 and 6, palatine gland; haematoxylin-eosin. Large mucous cells line the alveoli and occasional ducts with a flattened epithelium are present.

Plate 2

Figs. 1, 3 and 5, submaxillary, sublingual and labial glands respectively; haematoxylin-P.A.S. Intralobular ducts are relatively abundant, and appear as lightly stained areas within the P.A.S.-positive alveolar tissue. Figs. 2, 4 and 6, submaxillary, sublingual and labial glands, respectively; haematoxylin-eosin. Deeply stained serous cells are numerous in the submaxillary gland but infrequent in the sublingual and labial glands.

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THE DEVELOPMENT OF PAROTID SALIVARY SECRETION IN YOUNG GOATS

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The saliva of ruminant animals plays an important part in the control of the acidity and consistency of the contents of the reticulo-rumen sac. In the suckling ruminant, milk by-passes the reticulo-rumen by way of the oesophageal groove and flows directly into the abomasum, and the rumen remains small and contains only fluid until the animal starts to eat substantial quantities of solid food when 3–4 weeks old. A large salivary secretion is not necessary in the young animal that relies on milk for its sustenance, and indeed might tend to neutralize the acidity of the abomasal contents.

We know that in young calves the parotid glands secrete very little saliva (Krinitsin, 1940; Kay, 1958*b*) although in adult sheep these glands supply about half of the estimated total salivary secretion (Kay, 1960). More detailed information concerning the flow and composition of saliva in young ruminants was desirable, and a study of the development of parotid secretion in young goats was therefore undertaken. A preliminary account of this work has been published (Kay, 1958*a*).

METHODS

Acute experiments were conducted on twenty-four British Saanen goats of either sex and 2–487 days old. They were fed on milk by bottle until 2–3 months old, and in addition most received hay *ad lib.* and mixed meals from about 3 weeks. Examination of the rumen contents post mortem showed that the goats began to consume small quantities of the straw on which they were bedded when about 1 week old and that by 3 weeks the rumen was full of this material. Rumination was observed to begin when the goats were 2–3 weeks old.

Anaesthesia was induced and maintained by the intravenous infusion of NaCl solution 0·9 % (w/v), saturated with chloralose at 40° C. An attempt was made to maintain anaesthesia at such a level that weak flexor reflexes could be obtained from the limbs. In practice the depth of anaesthesia was sometimes sufficient to abolish flexor reflexes altogether, and at other times permitted brisk responses. Variation in the depth of anaesthesia over this range was associated with considerable variation in the resting rate of secretion of the innervated parotid gland, but had little influence on the flow from the reflexly stimulated gland or the resting gland after section of the parotid nerve, or on the flow caused by electrical stimulation of this nerve.

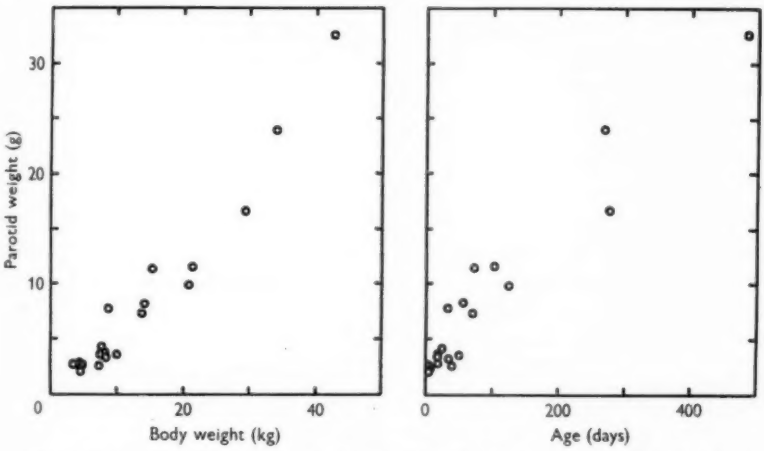
One or both parotid ducts were cannulated with polythene tubing immediately behind the papilla. The saliva was collected under oil and the rate of secretion was determined by

weighing the sample. The parotid nerve was isolated either beside the parotid duct or beneath the masseter muscle before it joined the duct. The peripheral end of the nerve was stimulated with a square-wave pulse applied by bipolar Perspex-shielded platinum electrodes. At the end of the experiment the parotid glands were dissected out, freed from extraneous tissue and weighed.

Sodium and potassium were estimated by flame photometry (Evans Electroselenium). Total CO_2 was estimated by the microdiffusion method of Conway (1950), inorganic phosphate by the method of Fiske & Subbarow (1925) modified by use of the reducing agent recommended by King (1951), and chloride by the potentiometric method of Sanderson (1952). Estimations were done in duplicate where volumes permitted. The accuracies achieved have been described previously (Kay, 1960).

RESULTS

Parotid gland weights. The combined weight of both parotid glands increased linearly both with body weight and with age. This is shown in Text-fig. 1. The slope of the regression of parotid weight (g) on body weight (kg) was 0·72 and that of the parotid weight (g) on age in days was 0·063.

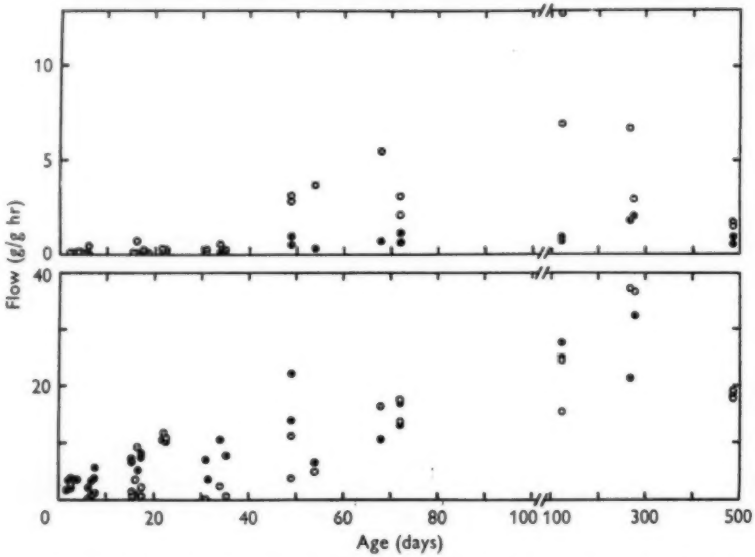


Text-fig. 1. The relation of the combined weight of both parotid glands to body weight and age.

Rates of secretion. In most experiments four samples of saliva were collected from one or from each parotid gland. The first was collected without the application of any stimulus to secretion, and the second while the thoracic oesophagus was distended with either a rubber tube or a balloon introduced through the mouth. Oesophageal distension has been shown to be powerful stimulus to parotid secretion (Kay & Phillipson, 1959). The parotid nerve was then cut, a third saliva sample was collected from the resting gland and a fourth sample during electrical stimulation

of the peripheral end of the parotid nerve. A swift and well maintained flow of saliva was obtained with a stimulus having a frequency of 7 pulses/sec and a pulse duration of 1 msec.

The rates of parotid secretion at different ages are shown in Text-fig. 2. The rates in goats less than 5 weeks old remained relatively low. The reflexly and electrically stimulated flows ranged from less than 1 to 12 g saliva/g parotid gland per hour and the flow from the resting gland after section of the parotid nerve did not exceed 0·1 g/g.hr. Frequently the



Text-fig. 2. Above, rate of secretion of parotid saliva at different ages by unstimulated glands; ○, parotid nerve intact; ●, nerve cut. Below, rate of secretion from glands stimulated reflexly ○, and electrically ●. The stimulus to reflex secretion was distension of the thoracic oesophagus.

flow from the resting gland stopped altogether. This was always due to a plug of mucus blocking the cannula, for when the obstruction was dislodged by gentle massage the flow recommenced. During the second and third months the rates of secretion gradually increased so that the parotid glands of goats more than 3 months old secreted at rates of 15–37 g/g.hr when reflexly or electrically stimulated and 0·6–2·1 g/g.hr from the resting gland after section of the parotid nerve.

In some goats it appeared that the parotid nerve did not contain all the secretory fibres that reached the gland, for section of this nerve did not always fully abolish the reflex responses. In the most extreme instance

encountered, in which oesophageal distension increased the flow from the innervated gland sixfold, the same stimulus still caused a threefold increase after the parotid nerve had been cut. In previous studies on sheep (Kay, 1958*b*) section of the parotid nerve had always wholly abolished reflex parotid secretion.

In four of the older goats one parotid gland was cannulated under Nembutal (pentobarbitone; Abbott Laboratories) anaesthesia by the method described by Ash & Kay (1959) on the day before the acute experiment. The following morning, before the induction of anaesthesia, each goat was placed in a recording stand and two samples of saliva were collected, the first immediately before feeding and the second while the goat was eating mixed meals. The excitability and rates of feeding of the goats varied considerably. In Table 1 the rates of secretion of these samples are

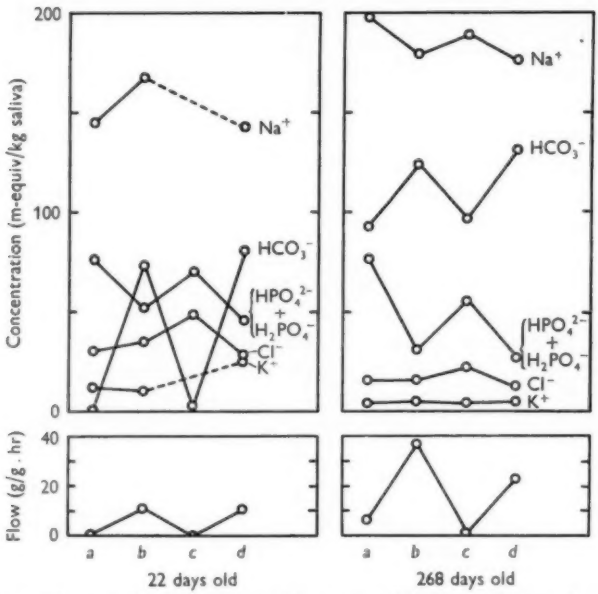
TABLE 1. The rates of parotid secretion in goats before and after anaesthesia

	Rate of secretion (g/g.hr)			
	Conscious		Anaesthetized	
Age (days)	Resting	Feeding	Resting	Reflexly stimulated
54	0·9	7·2	3·7	4·9
68	3·7	7·4	5·5	16·5
124	10·4	43·1	13·4	24·3
487	3·6	12·9	1·7	17·9

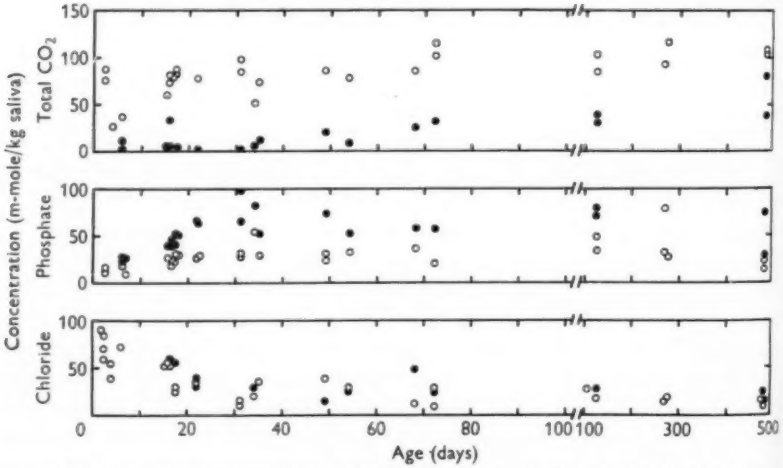
compared with those of the unstimulated and reflexly stimulated samples collected later under anaesthesia. Although there was a general correspondence for each goat in the rate of the resting flow when conscious with that of the resting flow when anaesthetized, and similarly for the rates of the reflexly stimulated flows when conscious and anaesthetized, this was not marked, possibly as a result of variation in excitability before anaesthesia and in the depth of subsequent anaesthesia.

Saliva composition. The composition of the saliva varied considerably with the rate of secretion (Text-fig. 3) and to a lesser extent with age (Text-fig. 4).

There was no consistent change in the concentrations of sodium or potassium with either rate of secretion or age. Total CO_2 concentration varied greatly with the rate of secretion; with few exceptions it remained below 40 m-mole/kg saliva when the flow was less than 1 g/g.hr and increased to 50–130 m-mole/kg at flows greater than 3 g/g.hr. Inorganic phosphate concentration, conversely, was high (25–100 m-mole/kg) at the slower rates of secretion and low (10–60 m-mole/kg) at the faster rates of secretion. There was rather less phosphate in the saliva of goats less than 3 weeks old than in that of older goats. Chloride concentration did not



Text-fig. 3. The variation in the composition of parotid saliva with rate of secretion in a young goat (left) and an adult goat (right). The four samples collected in each experiment were taken when the parotid gland was (*a*) unstimulated and innervated, (*b*) reflexly stimulated by distension of the thoracic oesophagus, (*c*) unstimulated after section of the parotid nerve and (*d*) during electrical stimulation of the peripheral end of the parotid nerve.



Text-fig. 4. The concentrations of bicarbonate, phosphate and chloride in the parotid saliva of goats of different ages. ○, flow > 3 g/g.hr; ●, flow < 1 g/g.hr.

vary consistently with the rate of secretion but fell from 40–90 m-mole/kg in goats less than one week old to 25–60 m-mole/kg at 1–3 weeks and 10–40 m-mole/kg in older goats.

The composition of saliva obtained from the conscious goats was similar to that of samples obtained later from the same goats under anaesthesia at comparable rates of secretion.

Histological appearance. Sections from the parotid glands of seven goats were stained with haematoxylin and eosin. In four of the goats, aged 106, 124, 276, and 487 days, the appearance of the gland was typically that of the adult ruminant (Pl. 1, fig. 3). The secretory tissue consisted of masses of serous secretory cells through which ran occasional intralobular ducts. The serous cells contained numerous cytoplasmic granules and their nuclei occupied only a small fraction of their volume. The parotid glands of two goats aged 6 and 17 days presented a wholly different appearance (Pl. 1, fig. 1). Intralobular ducts were numerous and the remaining volume of the gland was largely occupied by structures that were either smaller ducts or developing alveoli. The cells lining the smaller ducts and alveoli and filling the intervening spaces were smaller than the serous cells of the adult parotid and few or no granules were present in their cytoplasm. The parotid gland of a 68-day-old goat was intermediate in appearance (Pl. 1, fig. 2).

DISCUSSION

The parotid gland of the goat is physiologically and histologically immature at birth and only reaches maturity at about 3 months of age. The ability to secrete is especially poorly developed during the first 5 weeks of life, when there is almost no continuous secretion and the response to stimulation is only about one third of that of an equal weight of adult parotid tissue. In young calves also the parotid glands secrete slowly if at all (Epaneshnikov, 1935; Kay, 1958*b*) and it is possible that they develop less rapidly than in the goat for Kay & Phillipson (1959) found that the maximum rate of secretion by reflexly stimulated parotid glands (ml./g.hr) is considerably less in calves from 4 to 8 months old than in adult sheep. Epaneshnikov (1935) found that the onset of continuous parotid secretion in calves coincides with the change from milk to vegetable diet.

Nevertheless the composition of the parotid saliva in young goats, allowing for the slow rate of secretion, is similar to that of adult goats. It differs only in that a small part of the phosphate is replaced by chloride in goats less than 3 weeks old. Dancis, Grobow & Boyer (1957) found similarly that the chloride concentration of the mixed saliva of infants is considerably higher than that of older children, although it is not clear from their results whether the change in the chloride level is independent

of changes in the rate of secretion and in the relative contributions of different glands.

Histologically the alveolar cells of the young goat's parotid gland appear relatively undifferentiated and form a smaller proportion of the total glandular mass than in the adult. The period during which these cells become differentiated corresponds to the period during which the rate of secretion of the gland is increasing. Their small number and immaturity in the young animal thus probably account for the poor secretory performance of the gland. The occasional mucous cells and alveoli found in the parotid glands of young sheep by Bock & Trautmann (1914) were not seen in the present study.

Sufficient solid food was being consumed by the goats when 3 weeks old to fill their forestomachs and permit rumination. The onset of ruminal digestion introduces nervous and possibly humoral factors which may augment self-differentiation (Borghese, 1950) and inductive processes in determining the rate of growth and differentiation of the parotid gland. The chewing of solid food, rumination and the presence of digesta in the forestomach will cause reflex stimulation of the parotid gland from the sensory areas of the mouth, oesophagus and forestomach (Clark & Weiss, 1952; Kay 1958*b*; Ash & Kay, 1959) that will have been almost wholly lacking when only milk was consumed. The existence of a humoral factor influencing parotid secretion was claimed by Krinitsin (1940), who showed that the rate of secretion of the denervated parotid gland declines little in calves that are fed normally but almost ceased in calves that fasted for 5 days following bilateral vagotomy. He concluded that absorbed products of digestion normally regulate the rate of secretion of the denervated gland.

Calves may be weaned to solid food when only a few weeks old and in normal practice the transition from liquid to solid food is gradual. If the change is made abruptly when the calves are 2–5 weeks old digestive disorders such as bloat and diarrhoea sometimes occur, and the acidity of the rumen contents often falls to below pH 5 (T. R. Preston, personal communication). It seems possible that insufficient saliva is secreted at these ages to play its normal part in neutralizing the acidic products of rumen fermentation.

SUMMARY

1. The rate of secretion and composition of parotid saliva were studied in anaesthetized goats that were 2–487 days old.

2. In goats less than 5 weeks old there is very little resting secretion and the stimulated rates of secretion, per gram parotid tissue, are only about one third of the adult rate. The rates of secretion subsequently increase to reach the adult level in 3-month-old goats.

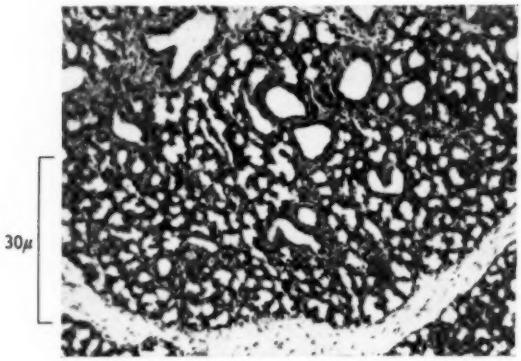


Fig. 1

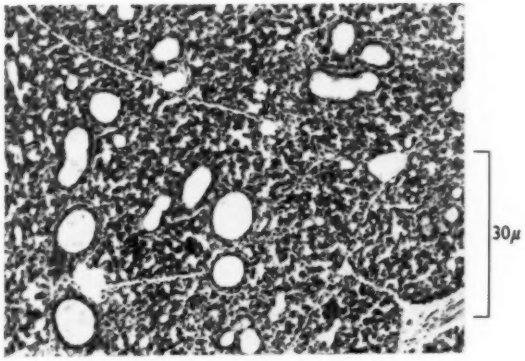


Fig. 2

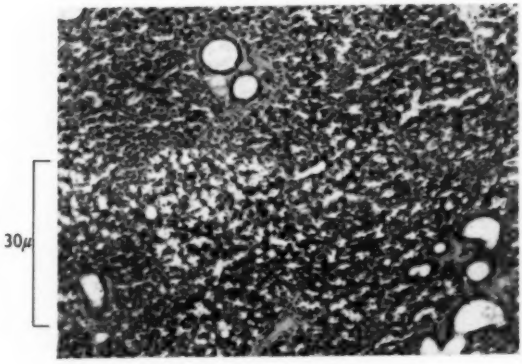


Fig. 3

(*Facing p.* 545)

3. The concentrations of bicarbonate and phosphate in the saliva vary considerably with the rate of secretion. The concentration of chloride is above adult levels for the first 3 weeks.

4. In goats less than 3 months old the parotid gland is histologically immature.

I wish to thank Mr D. Pollock for technical assistance and Mr B. F. Fell and Mr J. Bruce for helping to prepare and photograph the histological material.

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EXPLANATION OF PLATE

Sections of goat parotid glands, fixed in 10% formol-saline and stained with haematoxylin-eosin. Fig. 1, parotid gland of 6-day-old goat; fig. 2, parotid gland of 68-day-old goat; fig. 3, parotid gland of 106-day-old goat.

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A STUDY OF THE MECHANISM OF PASSIVE SENSITIZATION

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(*Received* 29 *July* 1959)

Isolated guinea-pig lung which is incubated with antibody becomes passively sensitized, and if it is subsequently treated with antigen it releases histamine (Mongar & Schild, 1957*b*). In the present work this system has been employed to investigate the passive sensitization reaction.

Antibodies are γ-globulins, and it seemed possible therefore that non-antibody γ-globulins would compete with them and thus interfere with passive sensitization. It was found, indeed, that γ-globulins inhibit, and under some circumstances reverse, passive sensitization of isolated guinea-pig lung. The effects of temperature, metabolic inhibitors and altered ionic environments on passive sensitization have also been studied. Other workers have in the mean time investigated independently the effects of γ-globulins on passive skin sensitization and on the passively induced Dale–Schultz reaction. Each of these investigations has already been briefly reported (Biozzi, Halpern & Binaghi, 1958; Halpern, Liacopoulos, Liacopoulos & Binaghi, 1958; Mongar & Schild, 1959).

METHODS

Preparation of lung tissue. Albino guinea-pigs weighing 400–600 g were killed by a blow on the head: the lungs were removed and in some experiments perfused through the pulmonary artery for 5–10 min until the effluent was clear. Longer-lasting perfusions, of 20–30 min, were carried out in those experiments in which the effect of perfusion on subsequent passive sensitization was studied. The lungs were cut into slices of about 5 mm thickness. The primary bronchi and patches of lung which had not been perfused free from blood were excluded. The slices were placed on two thicknesses of hard filter paper and cut into rods of $0{\cdot}8 \times 0{\cdot}8$ mm cross-section with the McIlwain tissue chopper (McIlwain & Buddle, 1953). The lung particles were suspended in about 500 ml. Tyrode solution, stirred for a few minutes, filtered on a coarse sintered-glass funnel and freed from excess liquid by gentle suction. They were then divided into 12–18 uniform samples of 0·1–0·2 ml. volume with the aid of a Perspex sampler previously described (Mongar & Schild, 1953).

Passive sensitization in vitro. The samples were suspended in 2 ml. of Tyrode solution containing either 2% rabbit antiovalbumin serum or 5% guinea-pig antiovalbumin serum, in 10 ml. tall beakers. The beakers contained a glass bead and were gently rocked in a thermostatically controlled bath at 38° C, or some other temperature, for periods varying from 15 min to 10 hr. In a few experiments the beakers were left at room temperature or at 4° C up to 50 hr with occasional shaking. In experiments lasting for more than 2 hr, chlortetracycline

(0·01 mg/ml.) was generally added; control experiments showed that this had no effect on passive sensitization.

Inhibitors of passive sensitization were allowed to equilibrate with tissue for 15 min before addition of antibody, except for γ-globulins and other proteins which were applied together with antibody. Before testing for histamine release with antigen the inhibitors were removed by repeated washing with Tyrode solution and suitable checks were made to ascertain that they did not produce persistent effects which might interfere with histamine release.

In some experiments passive sensitization was carried out *in vivo* by injecting 1 ml. guinea-pig antiovalbumin serum into the ear vein of guinea-pigs. The calculated ratio, antibody : non-antibody γ-globulin in the circulation immediately after intravenous injection was about 0·01.

Histamine release. The amount of histamine released by the antigen was used as a measure of passive sensitization. Histamine release and bioassay were carried out as follows. After sensitization the antibody solution was sucked off with a pipette fitted with a small cup plugged with cotton wool. The tissue was then washed with 1 ml. Tyrode solution to remove excess antibody and finally 2 ml. antigen solution (1 mg/ml. ovalbumin in Tyrode solution at 38° C) was added. This was left in contact with the tissue for 15 min, during which time histamine release is about 90 % completed. The antigen was always applied at 38° C even when sensitization was carried out at a different temperature. In such cases the tissue was first brought to 38° C for a minute or so and the antigen then added. Control experiments showed that lengthy incubation of tissue at a low temperature did not affect its ability to release histamine with the antigen. Thus samples of actively sensitized chopped lung which had been left standing in Tyrode solution for 4 hr at 0, 22 or 38° C released the same amount of histamine at 38° C afterwards.

Histamine determinations were carried out on the atropinized guinea-pig ileum by means of automatic assay apparatus, with 1 ml. jacketed organ baths and a technique of complete replacement of the bath fluid by the test solution (Boura, Mongar & Schild, 1954). As a rule all determinations were carried out on duplicate lung samples. After a preliminary assay the test solutions were diluted so as to be approximately equally active. They were then assayed in batches from 6 to 12, applied consecutively, and then in reversed order, flanked on both sides by three doses of the standard. The histamine equivalent of the test solutions was estimated by interpolation and assuming a uniform change of sensitivity with time. Each test solution was administered at least four times. To determine the histamine content of the lung particles they were heated in 10 ml. Tyrode solution for 5 min in a boiling water-bath and the supernatant assayed.

Materials used. Antiovalbumin serum from two rabbits injected with 100 mg ovalbumin at intervals of 3–4 days was used for passive sensitization. Nine injections were given; three intraperitoneally, three intravenously and three more intraperitoneally. The animals were bled one week after the last injection. The antibody content of the combined sera was 8 mg/ml. when titrated with ovalbumin by the procedure of Kabat & Mayer (1948). Guinea-pig antiovalbumin serum was obtained by injecting 20 mg ovalbumin subcutaneously in incomplete Freund's adjuvant. The animals were bled 3 months later. The serum contained 5 mg/ml. antibody. In one experiment rabbit γ-globulin containing 40 % antibody to pneumococcus type III polysaccharide was used (kindly supplied by Dr J. H. Humphrey).

Human γ-globulin, kindly supplied by Dr W. d'A Maycock of the Lister Institute of Preventive Medicine, contained 96 % γ-globulin and 2·5 % β-globulin (batch EG 105, fraction G 4). Human albumin obtained from the same source was about 99 % pure (batch EPA 133, fraction AP 3). The bovine γ-globulin used was a commercial sample obtained from Armour and Co. Ltd. (plasma fraction II, batch BF 0270).

RESULTS

Time course of passive sensitization: effect of temperature

Chopped lung was incubated with antibody for different times and then treated with antigen. Figure 1 (top curve) shows the amounts of histamine released after incubation of lung with antibody at 38° C for varying times. The releases increase at first linearly with time, reaching a maximum after about 3 hr. The rate and maximum of sensitization—in terms of lung histamine released per cent—varied considerably in different preparations, even though the same antibody was used (Table 1).

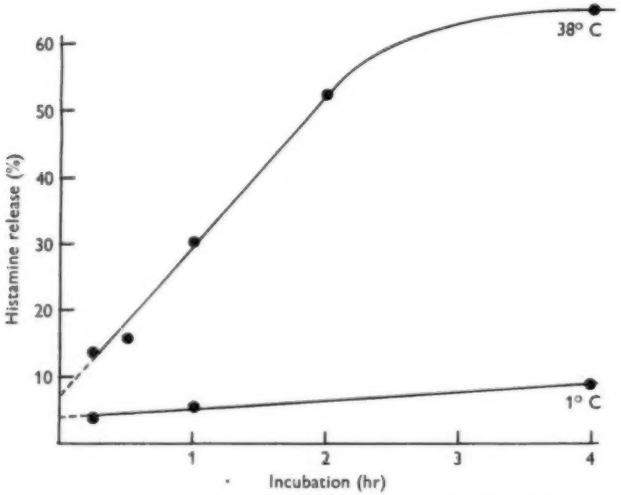

Fig. 1. Time course of passive sensitization of guinea-pig lung particles incubated with 2% rabbit antiovalbumin serum in Tyrode solution at 38 and 1° C. In this and subsequent figures the ordinate represents tissue histamine (%) released at 38° C when the lung was treated with antigen (ovalbumin 1 mg/ml.) after passive sensitization.

No histamine was released unless the tissue was incubated with antibody alone for some time. In experiments in which antigen was applied within 10 sec of antibody no significant histamine release occurred, although the ratio of concentrations of antigen and antibody was varied twenty-fivefold.

The influence of temperature on the rate of sensitization is shown in Fig. 1. It is seen that the initial rate at 1° C is only 6% of that at 38° C. The final sensitization achieved is however independent of temperature. Figure 2 shows that in order to achieve the same degree of sensitization

TABLE 1. Passive sensitization of non-perfused chopped guinea-pig lung particles with rabbit antiovalbumin serum; 0·2 ml. tissue incubated at 38° C with 2 ml. 2% serum in Tyrode solution for varying times (hr). Histamine release as per cent of tissue content

Expt. no.	Time (hr)	Histamine release (%)	Expt. no.	Time (hr)	Histamine release (%)
1	0	0·2	7	3	32
	¼	0·6		6	32
	½	1·3			
	1	4·2	8	1½	17, 19
	2	10·8		3	30, 32
	4	19·2		6	45, 46
2	½	2·3	9	½	10, 11
	1	3·0		1	14, 14
	2	7·5		2	22, 25
				4	29, 29
3	¼	1·0		6	17, 23
	½	1·3			
	1	3·5	10	½	3·1, 3·3
	2	6·8		1	6·6, 7·6
				2	22, 23
4	¼	0		4	22, 27
	1	1·3		6	24, 25
	4	9·7			
			11	1	19, 20
5	2	16		2·1	29
	4	29		3·5	36, 50
				5·3	43, 44
6	½	4·1			
	1	16			
	3	45			

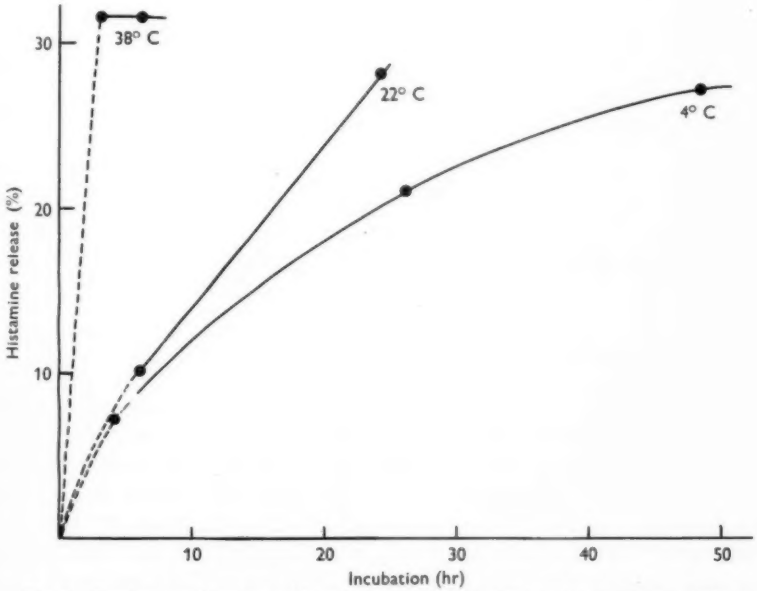

Fig. 2. Passive sensitization with rabbit antibody at different temperatures. The points at 22 and 38° C are from the same lung and those at 4° C from a different lung.

the following times are required: 2½ hr at 38° C, 25 hr at 22° C and 50 hr at 4° C.

In some other experiments lung tissue was incubated with antibody for a fixed time. Figure 3 shows histamine releases after incubation of lung with rabbit antibody for 4 hr at temperatures varying from 3 to 41° C. Incubation below 20° C produced little sensitization; between 30 and 40° C sensitization increased steeply, reaching a maximum at about 40° C.

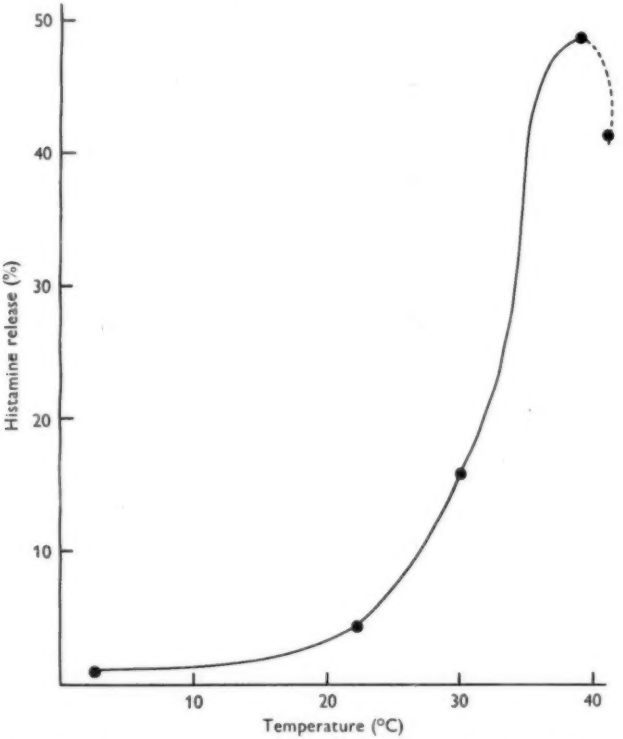

Fig. 3. Passive sensitization after 4 hr incubation of guinea-pig lung particles with rabbit antibody at different temperatures.

Incubation above 40° C produced a smaller histamine release, but this may have been due to damage to the anaphylactic mechanism (Mongar & Schild, 1957*b*) rather than to diminished fixation of antibody. Several experiments were performed, all showing a generally similar temperature dependence (Table 2).

In order to measure the temperature coefficient of passive sensitization the sensitization rate was measured at 30 and 35° C. From the slope of

the curves of histamine release against time of incubation Q_{10} values of 3·5–7·4 were calculated. These results are shown in Table 3. (In an earlier preliminary communication (Mongar & Schild, 1959) the Q_{10} was determined directly from the slope of the plot of histamine release against temperature, which gave the rather high value of 10.)

TABLE 2. Histamine release by antigen after passive sensitization of non-perfused guinea-pig lung with rabbit antibody at various temperatures

Expt. no.	Time (hr)	Temp. (° C)	Histamine release (%)	Expt. no.	Time (hr)	Temp. (° C)	Histamine release (%)
1	2	30·1	3·2	5	4	2·5	0·9
		33·3	5·9			22·3	4·4
		36·1	19			30·0	16
		38·2	34			38·7	48
		40·0	38			40·8	41
		42·0	25	6	3	0	3·1
2	4	0	1·1			20	7·2
		20	4·3			38	45
		38·5	53	7	3	20	3·8
3	4	10·7	5·4			38	32
		30·5	14·5	8*	2	24	2·4
		38·3	46			38	7·4
		42·0	6·9				
4	2	24	0·1				
		38	16				

* Sensitization with pneumococcus antibody.

TABLE 3. Temperature coefficient of passive sensitization

Temp. (° C)	Time (min)	Histamine release (%)	(%/hr)	Q_{10}
35·3	15	11·7	19·9	3·5
	60	26·6		
30·0	30	14·7	10·2	
	120	30·0		
35·0	15	19·0	21·4	5·6
	60	35·0		
30·0	30	24·5	9·0	
	120	38·0		
34·9	15	2·0	15·3	7·4
	60	13·5		
30·1	30	1·8	9·5	
	120	9·9		

Extrapolation to zero time of the sensitization curve seemed to reveal an initial rapid phase of sensitization preceding the linear phase (cf. Fig. 1). The rapid initial phase was particularly apparent when the lung had been previously well perfused. In experiments in which one lobe was thoroughly perfused with Tyrode solution whilst the other was not perfused the initial sensitization rate was always higher in the perfused lobe. Table 4

shows that passive sensitization after 15 min incubation with antibody was about twice as great in the perfused as it was in the unperfused lobe. This rapid initial phase occurred even at 0° C and was then followed by a slow increase in sensitization, as already described. Table 4 shows that histamine release after 2 hr incubation at 0° C was only about twice that after 15 min incubation. The initial rapid sensitization was however always much less than the maximum that can be achieved. Thus the histamine release after 2 hr incubation at 38° C was about nine times as great as that after 15 min at 0° C. (A rapid initial sensitization in the cold has also been observed by W. E. Brocklehurst, J. H. Humphrey & W. L. M. Perry (personal communication).)

TABLE 4. Effect of lung perfusion on sensitization. One lobe of lung was perfused for 20–30 min with Tyrode solution, the other was not perfused. Both lobes were then chopped up and incubated with antibody. Numbers indicate % histamine released with antigen

Incubation with antibody	15 min at 0° C		2 hr at 0° C		2 hr at 38° C	
Expt. no.	1	2	1	2	1	2
A Not perfused	2·3	3·2	4·7	6·1	58	48
B Perfused	5·0	6·0	8·5	10·1	50	55
B/*A*	2·2	1·9	1·8	1·7	0·9	1·1

Effect of metabolic inhibitors

The choice of metabolic inhibitors was limited to those which do not produce an irreversible impairment of the anaphylactic mechanism. Table 5 shows that none of the metabolic inhibitors used interfered with passive sensitization. Some of the substances used, e.g. phenol and cyanide, completely block the anaphylactic histamine release when applied together with the antigen (Mongar & Schild, 1957*a*). There is thus a marked contrast between the effects of metabolic poisons on passive sensitization and on the histamine-release mechanism.

Effect of ionic environment

In some experiments the ionic composition of the incubation solution was varied. Sodium edetate (0·05 %) was added to the Tyrode solution to bind Ca and Mg ions, or Tyrode solution was replaced by isotonic or hypertonic NaCl solution. In each case the lung tissue was thoroughly rinsed with Tyrode solution to remove the abnormal ions before testing with antigen. Table 6 shows that abnormal ionic environments had little or no effect on passive sensitization. Normal sensitization occurred in isotonic and in strongly hypertonic NaCl solution. Appreciable sensitization even occurred in sucrose solution. The calcium-chelating agent sodium edetate,

which completely inhibits the anaphylactic mechanism (Mongar & Schild 1958) was likewise without effect on passive sensitization.

TABLE 5. Effect of metabolic inhibitors on passive sensitization

Inhibitor	Concn. (mM)	Histamine release (%) Expt.	Control
Cyanide	5	46	29
	10	39	37
Azide	10	26	29
	50	26	29
Fluoride	2	32	29
	5	29	29
Dinitrophenol	2	32	37
Phenol	10	20	16

TABLE 6. Effect of abnormal ionic environments on passive sensitization

Modified solution	Histamine release (%) In modified soln.	In Tyrode control
Tyrode + sodium edetate 0·05%	19	15
NaCl (0·14 M) + KCl (0·003 M)	28	25
NaCl 0·15 M	26	25
0·9 M	9	16
1·0 M	19	15
1·7 M	6·5	16
Sucrose 0·3 M	15	25

Effect of γ-globulins on passive sensitization

Passive sensitization of guinea-pig lung by rabbit or guinea-pig antiovalbumin antibodies was strikingly inhibited by neutral γ-globulins (i.e. γ-globulins without antiovalbumin activity) either in pure form or in serum. The following combinations were used:

Passive sensitization by	Substance added
Rabbit antibody	(*a*) Rabbit serum
	(*b*) Human serum
	(*c*) Human γ-globulin
	(*d*) Human albumin
	(*e*) Bovine γ-globulin
	(*f*) Guinea-pig serum
Guinea-pig antibody	(*g*) Guinea-pig serum

All combinations produced inhibition except (*d*) and (*e*).

(*a*) *Rabbit antibody–rabbit serum.* The effect of 10% rabbit serum on passive sensitization by 2% rabbit antiovalbumin serum is shown in Fig. 4. In the controls nearly maximal sensitization was produced in 2 hr. In the presence of rabbit serum sensitization was much slower. No histamine release occurred after 1 hr incubation, after 2 hr it was only 20%

of the control and even after 6 hr the control value had not been reached.

(*b*) *Rabbit antibody–human serum.* Human serum also inhibits passive sensitization by rabbit antibody. In experiments similar to those in the preceding section, except that 10% human serum was used, the serum produced strong inhibition of passive sensitization after 1½ hr incubation (Table 7).

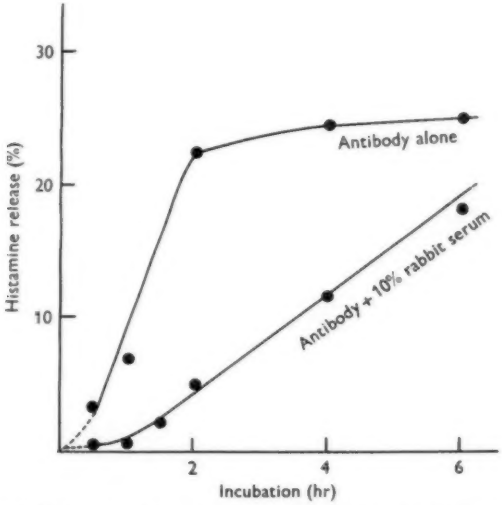

Fig. 4. Inhibition of passive sensitization with 2% rabbit antiovalbumin serum by 10% normal rabbit serum at 38° C.

TABLE 7. Effect of human serum and purified γ-globulin on passive sensitization. Guinea-pig lung tissue incubated with 2% rabbit antiovalbumin serum for 1½ hr at 38° C

Antibody in Tyrode plus	Histamine release with antigen (%)
Nil	19, 23
Normal human serum 10%	3·4, 4·6
Human γ-globulin 1 mg/ml.	8·3, 8·2
Human γ-globulin 4 mg/ml.	0·3, 0·5

(*c*) *Rabbit antibody–human γ-globulin.* Purified human γ-globulins are highly effective inhibitors, their activity depending on concentration. Figure 5 shows the effect of 1 mg/ml. and 10 mg/ml. human γ-globulin on sensitization by rabbit antibody. 1 mg/ml. γ-globulin produced an initial delay followed by sensitization at a normal rate, reaching a maximum after 6 hr; 10 mg/ml. caused practically complete inhibition for the duration of the experiment. In another experiment the following hist-

amine percentages were released with (i) rabbit antiovalbumin serum alone and (ii) rabbit antiovalbumin serum and human γ-globulin (1 mg/ml.).

	Histamine (%)					
Time (hr) ...	½	1	1½	2	4	6
(i)	11	14	—	23	29	20
(ii)	2·1	5·8	12	18	28	21

This experiment shows more clearly the initial delay produced by γ-globulin followed later by maximal sensitization.

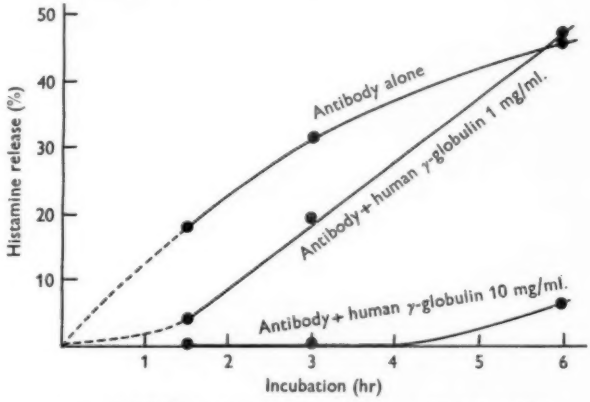

Fig. 5. Inhibition of passive sensitization with 2% rabbit ovalbumin serum by human γ-globulin at 38° C.

When human serum was used in the same experiment as purified human γ-globulin, a 10% concentration of serum produced an inhibitory effect corresponding to about 2 mg/ml. purified γ-globulin (Table 7). The actual concentration of γ-globulin in 10% serum is about 0·7 mg/ml. The serum thus appears to be rather more active than can be accounted for by its γ-globulin content. Nevertheless, purified serum albumin produced no inhibition, as is shown below.

(*d*) *Rabbit antibody–human albumin.* Human albumin (10 mg/ml.) produced no inhibition of passive sensitization, but on the contrary some potentiation (Table 8).

(*e*) *Rabbit antibody–bovine γ-globulin.* Bovine γ-globulin (1–10 mg/ml.) produced no inhibition of passive sensitization (Table 8).

(*f*) *Rabbit antibody–guinea-pig serum.* Guinea-pig serum inhibits sensitization of guinea-pig tissue. In one experiment 5% guinea-pig serum produced a threshold inhibition and 10% serum a 50% inhibition of passive sensitization by rabbit serum. In another experiment, shown in Fig. 6, 10 and 40% guinea-pig serum was used with rabbit antibody. The

10 % serum produced an initial delay followed by sensitization at the same rate as the control. The 40 % serum also produced a delay followed by sensitization at a diminished rate. The maximum seemed to be depressed in the presence of human serum but it is not clear whether this is a real effect, since after about 6 hr at 38° C the histamine-releasing activity of the controls diminished and approached that of the experimental samples.

TABLE 8. Effect of human albumin and bovine γ-globulin on passive sensitization. Guinea-pig lung tissue incubated with 2 % rabbit antiovalbumin serum for 2 hr at 38° C

Antibody in Tyrode plus:	Histamine release with antigen (%)
Nil	18, 23
Human albumin (10 mg/ml.)	31, 32
Nil	20, 21
Bovine γ-globulin (1 mg/ml.)	26, 28
Nil	26
Bovine γ-globulin (1 mg/ml.)	30
(4 mg/ml.)	30
(10 mg/ml.)	23

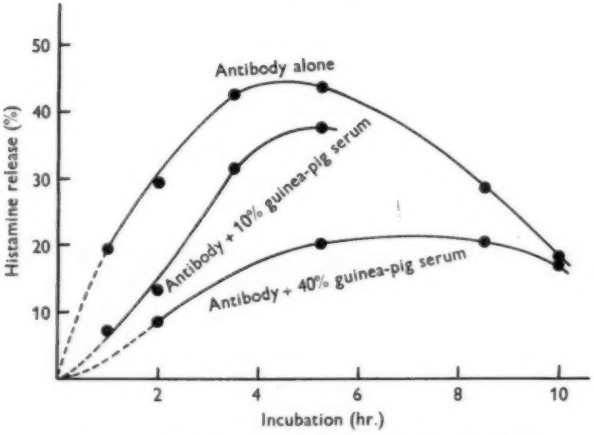

Fig. 6. Inhibition of passive sensitization with rabbit antibody by guinea-pig serum at 38° C.

(*g*) *Guinea-pig antibody–guinea-pig serum.* This corresponds to the conditions normally prevailing during active sensitization. The effect of guinea-pig serum on sensitization with guinea-pig antibody is shown in Fig. 7. A 5 % concentration of guinea-pig antiovalbumin serum was used as the antibody, diluted either with normal guinea-pig serum or with Tyrode solution. The normal serum produced an initial inhibition; e.g. after 1 hr incubation the sample in serum released 10 % histamine whereas the Tyrode control released 36 %. The difference between the two

samples became progressively less and after 4 hr had completely disappeared. After 5½ hr incubation the serum sample was more active than the control, possibly owing to unspecific deterioration of the control kept in Tyrode solution.

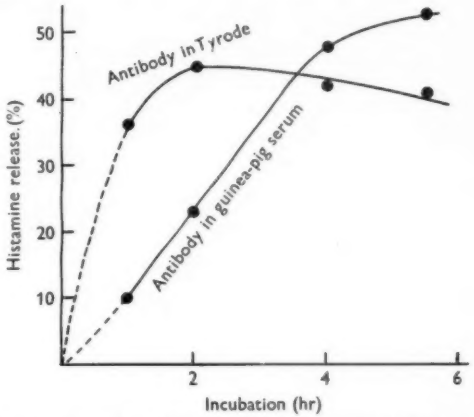

Fig. 7. Inhibition of passive sensitization with guinea-pig antibody by normal guinea-pig serum at 38° C.

Effect of γ-globulins on maximal sensitization

In the preceding experiments an effect of γ-globulins on the sensitization maximum was not clearly demonstrable owing to deterioration of the tissues after prolonged incubation at 38° C. Some experiments were therefore carried out at lower temperatures, at which spontaneous deterioration is less pronounced. Figure 8 shows an experiment carried out at 4° C in which sensitization by rabbit antibody was produced in the presence of human γ-globulins. It is seen that the sensitization maximum was reasonably well maintained at this temperature. In the presence of γ-globulin 4 mg/ml. and 10 mg/ml. the rate of sensitization and the sensitization maximum were diminished.

Reversal of sensitization by γ-globulins

We also investigated whether an established sensitization can be reversed by γ-globulin in either actively or passively sensitized lung.

Passive sensitization in vitro by rabbit antibody. Guinea-pig lung was sensitized by incubation with rabbit antibody for 2 hr. The antibody was then removed and the sample further incubated with 10 mg/ml. human γ-globulin, whilst controls were kept in Tyrode solution. The samples were tested 1, 2 and 3 hr later for release of histamine by antigen. Figure 9 shows that incubation with γ-globulin produced a progressive loss of

sensitization, whereas in the controls sensitization actually increased. Thus, after 1 hr incubation with γ-globulin histamine release was reduced to 40 % of the controls and after 3 hr to 4 %. In another similar experiment histamine release was reduced to 29 % of the control value after 2 hr incubation with γ-globulin. In an otherwise similar experiment in which

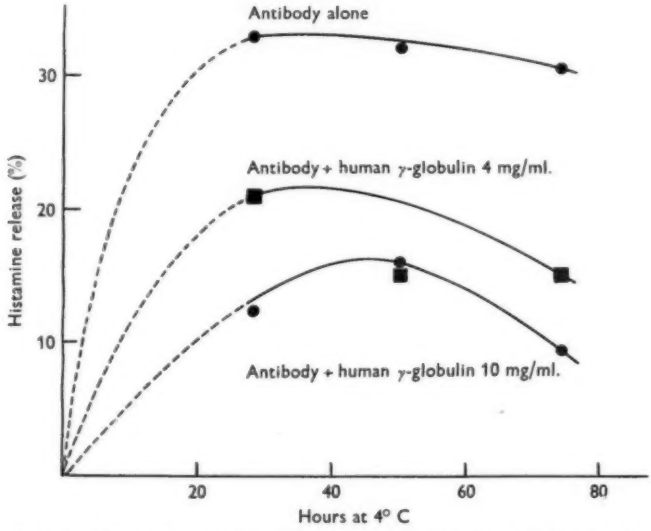

Fig. 8. Inhibition of passive sensitization with rabbit antibody by human γ-globulin at 4° C; note depression of sensitization maximum.

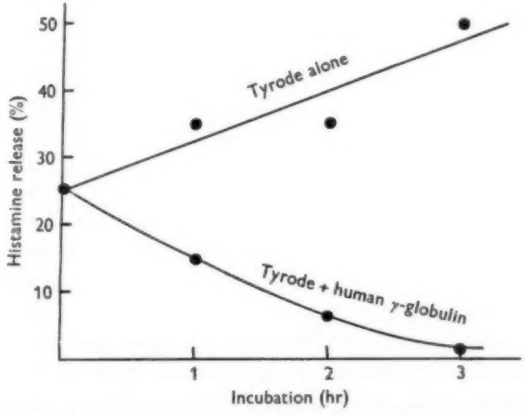

Fig. 9. Reversal of passive sensitization. Lung was first incubated for 2 hr with rabbit antibody; the antibody was then replaced by human γ-globulin (10 mg/ml.) at 38° C and incubation continued for the times indicated.

incubation with γ-globulin was carried out at 1° C instead of 38° C a smaller but quite definite reversal of sensitization occurred, histamine release being reduced to 56 % of control after 2 hr.

Passive sensitization in vitro by guinea-pig antibody. Sensitization of guinea-pig tissue by homologous antibody proved less easily reversible than by heterologous antibody. Table 9 shows that human γ-globulin (1 and 10 mg/ml.) produced no reversal of passive sensitization by guinea-pig antibody, but undiluted guinea-pig serum produced a partial reversal. Thus guinea-pig antibody could be displaced from homologous tissue by guinea-pig serum but not by human γ-globulin.

TABLE 9. Effect of incubation (3 hr at 38° C) with human γ-globulin or guinea-pig serum or Tyrode (control) on sensitization of guinea-pig lung previously incubated (1½ hr at 38° C) with guinea-pig antibody

	Histamine release (%)	
Incubation with:	Expt.	Control
Human γ-globulin, 1 mg/ml.	29, 31	30
10 mg/ml.	25, 34	
Guinea-pig serum, 10 %	30, 32	34, 35
100 %	14, 18	

TABLE 10. Attempted reversal of active sensitization

Incubation soln.	Time (hr)	Temp. (° C)	Histamine release (%) Expt.	Control
Human γ-globulin (10 mg/ml.)	3	38	34, 46	34, 35
	5	38	20, 31	24, 26
Guinea-pig serum (100 %)	4	38	22, 22	21, 22
	22	0	24, 25	35, 36

Active sensitization. Neither human γ-globulin nor guinea-pig serum reversed active sensitization. Table 10 shows that actively sensitized lung incubated at 38° C with human γ-globulin or guinea-pig serum released as much histamine as the controls kept in Tyrode solution. After incubation for 22 hr at 0° C there was possibly some slight reversal.

Effect of duration of contact with antibody. The preceding findings suggested that antibody is fixed more firmly in active than in passive sensitization. The following factors might be involved:

(1) Duration of contact with antibody.

(2) Route of access of antibody, e.g. by way of the blood *in vivo* or by diffusion *in vitro*.

(3) Source of the antibody, whether autologous or homologous.

These factors were investigated. In one experiment lung tissue was incubated with guinea-pig antibody *in vitro* for 1, 2 or 4 hr and then with normal guinea-pig serum for 1 hr. Table 11 shows that the sensitization for 1 hr was more completely reversed than longer-lasting sensitizations.

In another series passive sensitization was produced *in vivo* by intravenous injection of guinea-pig antibody into guinea-pigs. The animals were killed at various times after injection; their lungs were perfused with Tyrode solution to remove circulating antibody, and chopped up. Some of the lung samples were then incubated with guinea-pig serum for 1 (or 4) hr and others in Tyrode solution as controls. (In some experiments the

TABLE 11. Effect of duration of *in vitro* passive sensitization on reversal by incubation in guinea-pig serum for 1 hr at 38° C

Time of sensitization (hr)	Histamine release (%)		
	Expt. (*a*)	Control (*b*)	*a*/*b*
1	6·8, 7·8	18, 19	0·37
2	6·8, 9·5	12, 13	0·66
4	4·4, 5·2	8, 8	0·58

TABLE 12. Guinea-pigs were injected intravenously with antibody and killed after varying times (t_{sens}). Their lungs were perfused with Tyrode and subsequently either perfused with guinea-pig serum (*Per.*) or chopped and incubated with serum (*Inc.*) or with Tyrode (*Con.*) for 1 or 4 hr (t_{rev}). Numbers indicate tissue histamine (%) released by antigen. The last column gives the average histamine release after treatment with serum as a fraction of the control (*Residual sensn.*)

t_{sens} (hr)	t_{rev} (hr)	*Per.*	*Inc.*	*Con.*	*Residual sensn.*	
½	1	16, 16, 18	9·2, 9·6	43, 50	0·30	
1½	1	37, 41, 42	41, 49	51, 62	0·74	
3½	1	—	55, 59	65, 68	0·95	
	1	—	35, 47	28, 45		
17	1	—	18, 18	25, 27	0·74	0·97
			46, 47	58, 59		
	4	—	18, 21	16, 17	1·20	
			40, 53	37, 40		
66	1	—	18, 31	29, 35	0·75	1·02
			44, 49	63, 64		
	4	—	26, 36	22, 26	1·28	
			46, 55	36, 43		

lung was perfused with guinea-pig serum for 1 hr instead of incubation with serum.) Table 12 shows that already 30 min after injection of antibody the lungs had become strongly sensitized, but at this stage the sensitization was almost completely reversed by incubation with guinea-pig serum. Within 1½ hr of injection guinea-pig serum produced only a slight reversal of sensitization, and within 3½ hr no reversal at all. It can be concluded that the main factor determining reversibility is duration of contact with antibody. Route of access and source of antibody are not of primary importance.

DISCUSSION

It has been shown that non-antibody γ-globulins inhibit passive sensitization of guinea-pig tissues by antibody γ-globulins. Depending on experimental conditions, they either delay passive sensitization or diminish the maximum or reverse an already established sensitization. These effects are highly specific. The guinea-pig's own γ-globulin is a more effective inhibitor than human γ-globulin, whereas bovine γ-globulin is ineffective. Albumin is also ineffective. Our observations are in general agreement with the findings of Halpern *et al.* (1958) concerning inhibition of sensitization of smooth muscle by γ-globulins. The most likely explanation of both sets of observations is that there is competition between antibody and other globulins for receptor attachments on the cell surface.

These findings are relevant to the problem of cellular fixation of antibody. Although after the demonstration that anaphylaxis can be obtained in isolated smooth muscle (Schultz, 1910) even after freeing the preparation from blood by prolonged perfusion (Dale, 1913) the cellular theory of anaphylaxis has become generally accepted, it has been contended against it (Freund & Witney, 1928) that the antibody may not be attached to the cell but simply trapped in the intracellular spaces, so that the reaction between antigen and antibody would be occurring in solution. According to this view the reaction is a homogeneous reaction rather than a heterogeneous reaction on the cell surface; but if this were so the specific interference produced by other γ-globulins would be difficult to account for. It would be necessary to explain why normal guinea-pig γ-globulin present in solution interferes with the antigen-antibody reaction, yet bovine γ-globulin fails to do so. If on the other hand only a limited number of sites were available on the cell surface, capable of taking up γ-globulins of a special kind, the interference would become explicable.

It should be emphasized that our findings are based on measurements of histamine release and are thus strictly speaking relevant only to the binding of antibody to histamine-releasing cells, probably mast cells (Mota, 1959). It is possible that only mast cells exhibit specificity in globulin uptake, since Humphrey & Mota (1959) have shown that the gross tissue uptake of γ-globulins does not depend on their sensitizing properties.

It is necessary now to consider in more detail whether a simple competition hypothesis can account for the antagonism between γ-globulins. A hypothesis of this kind would explain a delay in the rate of sensitization and a depression of the maximum, but it would not account for the reversal of an established sensitization, since the rate of desorption of antibody from its receptors would not be expected to depend on the concentration of reactants in solution. Reversal of sensitization should occur as rapidly

in the absence as in the presence of γ-globulin. In order to account for the more rapid reversal in the presence of γ-globulin it is necessary to postulate a more complex scheme, e.g. one involving several attachments of each antibody molecule with receptors. Consider a series of consecutive reactions:

$$A + R \rightleftharpoons AR,$$
$$AR + R \rightleftharpoons ARR,$$
$$ARR + R \rightleftharpoons ARRR \ldots,$$

where A is the concentration of antibody, R that of free receptors, and AR, ARR, etc., those of antibody bound to one, two or more receptors. The rate of desorption would now depend on the concentration of the reactant AR. If by the introduction of neutral globulin the number of free receptors R were reduced, the reaction would tend to shift to the left and the concentration of AR, and hence the rate of desorption, would be increased.

This scheme could also account for the finding that passive sensitization becomes less reversible with time, since the reaction would tend in time to shift towards multi-attachment and in this way to become less easily reversible.

The fraction of receptors which has to be occupied by antibody to produce sensitization is probably small. After equilibrium has been established the fraction of receptors occupied by antibody would be expected to be equal to the ratio of antibody to non-antibody γ-globulin in the circulation. This should apply to the guinea-pig's own antibody, which is presumably indistinguishable by the cells from its other γ-globulins. Antibody from other guinea-pigs can be expected to have less affinity and hence to be adsorbed in a lower ratio than that present in the circulation. In the *in vivo* sensitization experiments it was found that an amount of homologous antibody equal to about 1 % of the γ-globulin in the circulation produced strong sensitization when given intravenously. The sensitization occurred in half an hour, before receptor equilibrium was reached. The fraction of receptors occupied by antibody at this stage must have been well below 1 %.

Passive sensitization has a high temperature coefficient and in this respect resembles the anaphylactic reaction, but in other respects the two reactions differ. The anaphylactic reaction requires calcium and is probably enzymic, whereas the passive sensitization reaction does not require calcium and is probably not enzymic, since it is unaffected by a variety of enzyme inhibitors. The high activation energy of the passive sensitization reaction may be required in the unfolding of the antibody γ-globulin molecule before it can be adsorbed on to the cell surface, or for the prior

desorption of non-antibody γ-globulin attached to the cell. The latter seems more probable in view of the finding that lung which has had some antibody removed by perfusion with Tyrode solution became partially sensitized at a rapid rate even at 0° C. The very high temperature coefficients observed in some experiments may on the other hand be due to the fact that in those experiments most receptors were initially occupied by non-antibody γ-globulins.

SUMMARY

1. The mechanism by which isolated chopped guinea-pig lung becomes passively sensitized by incubation with antibody *in vitro* has been studied by using the histamine release reaction with antigen as a measure of sensitization.

2. Passive sensitization increases with time of incubation and at 38° C becomes maximal in 2–4 hr. Passive sensitization has a high temperature coefficient but is not completely abolished even at 0° C. It is not inhibited by a variety of enzyme poisons nor by alterations in the ionic composition of the incubation medium, including complete absence of calcium and magnesium.

3. Passive sensitization is inhibited by the presence of non-antibody γ-globulin in the incubation mixture. The main effect of γ-globulin is a reduction of the rate of sensitization; under certain circumstances the maximum is also depressed and an already established sensitization reversed. The inhibitory effect is specific. It is produced by guinea-pig, rabbit and human sera, and by purified human γ-globulin, but not by purified bovine γ-globulin nor by human serum albumin. Reversal of passive sensitization could only be demonstrated within a short time of passive sensitization. In actively sensitized preparations no reversal was obtained.

4. These results suggest the following conclusion concerning the mechanism of the passive sensitization reaction. The reaction is non-enzymic with a high energy of activation required in the adsorption of antibody γ-globulin and the desorption of non-antibody γ-globulin. Antibody and non-antibody γ-globulins compete for specific cellular attachments, each γ-globulin molecule being attached to a number of receptors. Only a small fraction (less than 1 %) of receptors needs to be occupied by antibody γ-globulins for full sensitization.

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EFFECTS OF PHENOL ON NERVOUS CONDUCTION

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In therapeutics phenol has been used dissolved in glycerol as ear-drops and as an ointment to stop itching; it has also been used in aqueous solution to destroy nerve fibres, such as those of the sympathetic chain and those growing in an amputation neuroma. A further use for phenol was introduced by Maher (1955). He reported that the chronic and severe pain occurring in some cases of cancer could be relieved by the intrathecal injection of phenol dissolved in glycerol; later (1957) he used phenol dissolved in Myodil (ethyl iodophenylundecylate; Glaxo Laboratories). The therapeutic procedure consists of introducing by spinal puncture phenol dissolved in Myodil or glycerol and allowing it to make contact with the spinal roots in which conduction of impulses giving rise to the pain is occurring. Maher found that solutions containing between 5 and 10% phenol gave a satisfactory therapeutic result. Non-aqueous solvents, immiscible with cerebro-spinal fluid, were used, so that the effective concentration of phenol could be confined to the nerve roots with which the solvent makes contact.

When using this technique to treat patients, we have observed that there are two effects, a temporary or local anaesthetic effect, and a chronic effect. Within 1–2 min of the injection of the solution a loss of sensibility and an all but complete local paralysis develops; both diminish greatly within 15 min. The chronic effect consists of the relief of the pain due to the cancerous invasion, with no or almost no permanent impairment of sensibility or movement to be found on clinical examination.

Experiments have been undertaken to examine more critically the effects of phenol in solution in Myodil and glycerol, both the immediate effect on conduction in anterior and posterior nerve roots and any chronic effects which might become manifest in time and be demonstrable by histological methods. The results of the former investigation are reported here; those of the latter will be reported in a subsequent paper. Some of the results of both investigations have been shown to the Physiological Society

(Nathan, Sears & Smith, 1958). Some effects of phenol on nervous conduction in acute experiments in the cat have also been described by Iggo & Walsh (1959).

In the present paper the effect on conduction of phenol dissolved in Myodil has been compared with its action in aqueous solution and both have been compared with that of procaine.

METHODS

To obtain information which might be useful for understanding the action of phenol in Myodil or glycerol, the experiments were designed to mimic the therapeutic situation in man. They consisted of stimulating peripheral nerve and recording from anterior and posterior spinal roots of the cat, before and at various periods after the application of phenol solutions to the roots.

All experiments were performed on cats anaesthetized with Nembutal (pentobarbitone; Abbott Laboratories, 35–40 mg/kg). Various hind-limb nerves were dissected free and submerged in liquid paraffin (37–39° C) in a pool formed by drawing up skin flaps to a metal ring. The anterior and posterior roots from which recordings were made were exposed by means of a lumbosacral laminectomy. The roots were divided as close to the spinal cord as possible, labelled with coloured thread, then left in the c.s.f. in the floor of the trough formed by the laminectomy.

Recording had to be made from the roots in air and not in paraffin oil, as paraffin is not only miscible with Myodil but also acts as a solvent for phenol. Spinal roots being devoid of perineurium are liable to dry in air, and are also particularly susceptible to mechanical damage. To ensure stable conditions, a piece of card, slotted so as to admit the electrodes and covered with cotton-wool, was used to cover the laminectomy wound. In this manner an enclosed chamber was formed into which moistened warmed oxygen was passed; the recording electrodes were set deep in this chamber, so that, being surrounded by the tissues of the cat, they tended to remain at the correct temperature. The laboratory temperature was kept high, being between 25 and 30° C, and the cat's rectal temperature was kept at 39° C. Control experiments showed that these precautions were adequate for obtaining a preparation that remained stable for the duration of an experiment.

The conduction rate of the various fibre groups was determined by measuring the change in latency of the components of the compound action potential, when the stimulating cathode was moved a measured distance along the peripheral nerve. This method was essential, for at the stimulus intensities needed for stimulating the small fibres, repetitive firing of the larger A fibres might occur, producing a low-amplitude later wave not easily distinguished from that of the more slowly conducting fibres of the A group. At the end of the experiment the distance between stimulating and recording electrodes was remeasured when the nerves and roots were dissected out for this purpose. The conduction rate of the peak of each component of the compound action potential was then calculated.

Substances applied to roots. Myodil is an oily substance, immiscible with c.s.f.; when placed in the laminectomy trough it can be kept as a large discrete globule surrounding the root around which it is placed; it can easily be removed by means of a syringe and needle. The various concentrations of phenol in Myodil are given here in g/100 ml. The aqueous solutions were made up in Ringer–Locke solution, buffered to pH 7·4; the addition of phenol in the concentrations used did not alter the pH. All solutions were applied at the temperature of the c.s.f. in the laminectomy trough, and the Ringer–Locke solution used for washing the roots was also applied at that temperature.

Stimulation and recording. Stimulation was effected by means of condenser discharges from a thyratron stimulator fed through an isolating transformer to platinum electrodes;

the repetition frequency was 1/sec. Action potentials were recorded monophasically from the roots, amplified, and displayed on a dual-beam oscilloscope; the second beam carried the time scale. The signals were also monitored on a loudspeaker.

The optimum conditions for recording the A and C action potentials are different and so two different preparations were used.

A fibres preparation. Stimulating electrodes were placed on the sciatic, tibial, or sural nerves and recording was made from the anterior or posterior roots of the 6th or 7th lumbar or 1st, 2nd, or 3rd sacral roots. It was found that at a distance between the stimulating and recording electrodes of about 80 mm the γ and the δ components are formed into well defined peaks, separate from the $\alpha\beta$ spike and not greatly reduced in amplitude by temporal dispersion.

C fibres preparation. All C fibres referred to in this paper are dorsal root C fibres. The same preparation was not usually suitable for recording from A and from C fibres, for at the conduction distances optimal for A fibre recording the C action potential is not visible as a wave owing to temporal dispersion. It was found that a conduction distance of 12–15 mm gave a good display of the C action potential. This was obtained by both stimulating and recording from a posterior root. The distance that we found to be optimal in pilot experiments is about half that which Gasser (1950) found suitable for recording the C action potential in peripheral nerves. A possible reason for the difference is that the diameter of the C fibres of the root central to the ganglion is half that of the fibres peripheral to the ganglion, a fact shown by Gasser (1955); the consequent decrease in conduction velocity would result in greater temporal dispersion over the longer distance.

The C action potential has to be displayed on the slowly changing phase of the A after-potential. At the amplification required to record the C action potential the initial phase of the after-potential may be distorted by the antecedent high voltage A spike overloading the amplifier; but this does not affect the configuration of the C action potential.

Thus in the C fibres preparation both recording and stimulating electrodes were placed on the posterior root. Long filaments of the longest roots (usually the 1st or 2nd sacral) were cut through as close as possible to the foramina at one end and to the spinal cord at the other. These filaments were set up on the stimulating and recording electrodes which were placed deep in the laminectomy trough.

In order to examine the effects of a solution on both the A and the C fibres simultaneously, the following method was used. A long filament was cut free from the rest of the posterior root and was placed on the electrodes as described for C fibres; the records made from this filament served as a control, by providing evidence that this root contained C fibres, and that their action potential was recordable at the conduction distance used. The solution of phenol was then applied to the rest of the root which remained *in situ*, and records were made as described for A fibres. After a certain duration of application of the solution, a further filament of this root was removed; this filament was placed on the electrodes as described for C fibres. Thus the A action potential was recorded with the nerve and root *in situ* before and after the application of the phenol solution; and the C action potential of this root was recorded from a sample removed before the phenol solution was applied and from a second sample removed from the root after the application of the solution.

Method of applying phenol in Myodil

A fibres preparation. The solutions to be tested were placed in the laminectomy trough so that they covered the peripheral part of the root from which recording was being made; the length of nerve root in the solution was about 5 mm. The proximal recording electrode was placed so that it was more than 10 mm away from the part of the root immersed in the solution.

C fibres preparation. The solution was placed in a wire loop encircling the root midway between the stimulating and the proximal recording electrodes.

Method of applying aqueous solution

A fibres preparation. A Perspex chamber was made (Fig. 1). This chamber was placed in the laminectomy trough so that the root, divided from the spinal cord at its proximal end, could be passed through the groove D, then through the bath B, and through another small groove, on to the platinum electrodes E. The lid L was placed on the chamber and secured by a ligature. The root passing through the groove was sealed in by soft paraffin, to allow the part of the root on the recording electrodes to be covered with paraffin oil. The solutions were passed through the tubes I and O.

C fibres preparation. The method was either the same as for Myodil and glycerol solutions or else the aqueous solutions were applied on a pledget of cotton-wool encircling the root.

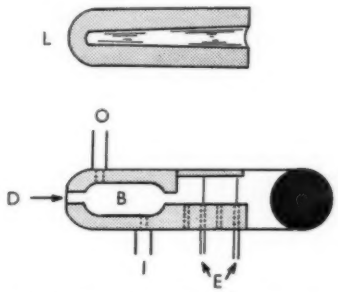

Fig. 1. Plan view of Perspex chamber.

RESULTS

Myodil and glycerol, the substances used as solvents for the phenol, were tested for any effects on nervous conduction; it was demonstrated that they had no effect during a period of 30 min. As the experiments with phenol dissolved in glycerol gave similar results to those in which it was dissolved in Myodil, they will not be reported upon separately. Most of the experiments were carried out with a 5 % solution of phenol in Myodil, which will be reported upon first; then will be described the effects of stronger and weaker solutions, the effects of phenol in Ringer–Locke solution, and finally the effects of procaine.

Phenol in Myodil

A 5% solution of phenol in Myodil blocks conduction, though recovery from the block can occur. The effects on the α and γ fibres of the A group of an anterior root are illustrated in Fig. 2 and on the C fibres of a posterior root in Fig. 3.

In the experiment illustrated in Fig. 2, 5 % phenol in Myodil was applied to the 7th lumbar anterior root. The α action potential of the fibres was travelling at 85–90 m/sec and that of the γ fibres at 33–34 m/sec. (Throughout this paper the nomenclature introduced by Erlanger & Gasser (1937)

has been used.) It will be seen that 135–140 sec after application of the solution the size of the γ wave is very greatly reduced, whereas that of the α wave is only moderately reduced. By 8 min, in this experiment, the γ wave had disappeared; by 20 min that of the α fibres had gone down to $12\frac{1}{2}\%$ of its original height. After 22 min the solution was removed and

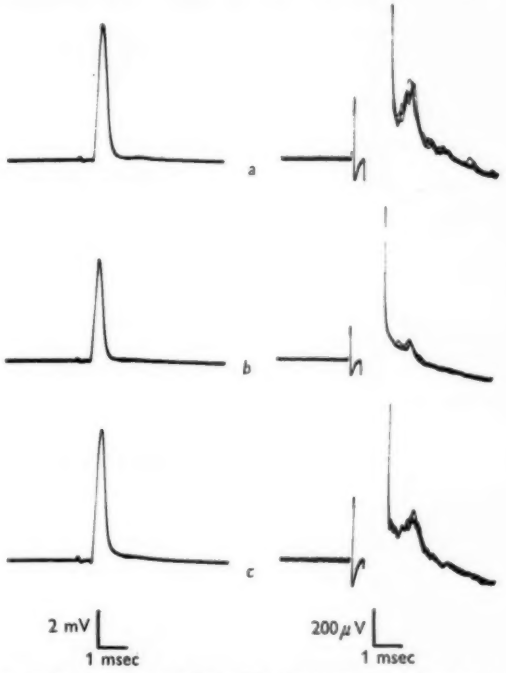

Fig. 2. Effect of 5% solution of phenol in Myodil on conduction in A fibres of an anterior root. Left column at low amplification to show fast conducting fibres (α range), right column at high amplification to show slowly conducting fibres (γ range). Each record consists of 5 superimposed responses. *a*, before application of solution; *b*, 135–140 sec after application; *c*, 10 min after removal of solution and washing.

the roots were washed with Ringer–Locke solution. Ten minutes after the removal the height of the α wave had returned to its original size; by 30 min after removal that of the γ fibres had not returned to more than 70% of its original size.

In the experiment illustrated in Fig. 3, 5% phenol in Myodil was applied to the 7th lumbar posterior root. The C action potential is seen superimposed on the A after-potential; Fig. 3*b* demonstrates the action potential at a strength of stimulation subliminal for C fibres. It will be seen that

within 20 sec the size of the C action potential had diminished considerably; at this time there was almost no effect on the A fibres, as may be judged by the amplitude of their after-potential. At 50 sec the C action potential was seen to be considerably diminished, at 1 min 5 sec it was still

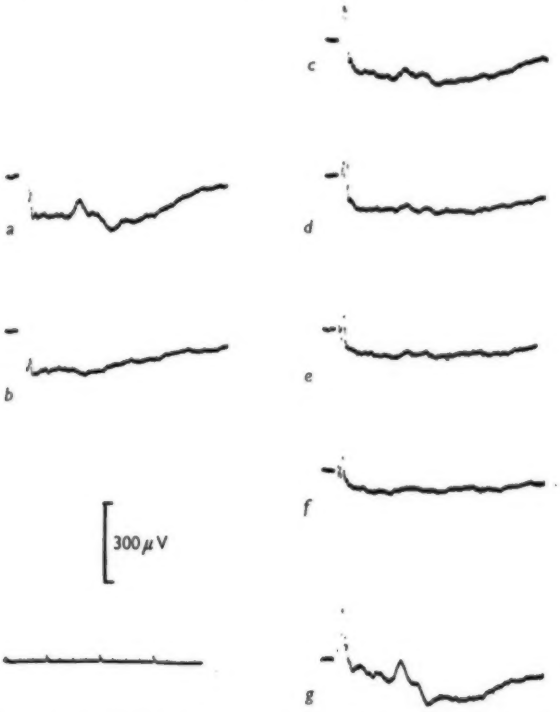

Fig. 3. Effect of 5 % solution of phenol in Myodil on conduction in C fibres of a posterior root. *a*, before application of solution, stimulus maximal for C fibres; *b*, stimulus subliminal for C fibres to show after-potential of A fibres; *c*, *d*, *e*, *f*, 20, 50, 85 and 105 sec after application; *g*, 13 min after removal of solution and washing. The time scale shows 1, 5 and 20 msec intervals.

just visible, and by 1 min 45 sec it had completely disappeared. The solution was removed 2 min after application and the root was washed. Sixteen minutes after removal of the solution, a large proportion of the C fibres were again conducting.

From the total evidence of thirty experiments it is concluded that a 5 % solution of phenol in Myodil blocks conduction in the A and C fibres. This blocking occurs differentially, affecting small fibres more rapidly than large. For example, within 4 min of applying a 5 % solution the

γ fibres were completely blocked; but at this time the α action potential had diminished by only 25%. In three experiments all the C fibres were blocked within 30 sec of application.

The following statements concerning recovery from blocking with a 5% solution are based on thirty experiments. With periods of application up to 50 min, some of the α fibres always recovered; with periods of application up to 10 min, recovery of all the α fibres was the rule. Complete recovery of all the γ fibres never occurred after application of the solu-

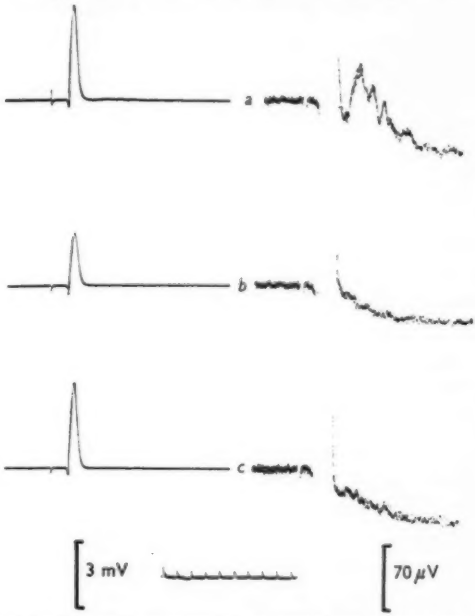

Fig. 4. Effect of 7·5% solution of phenol in Myodil on conduction in myelinated fibres of a posterior root. Each record consists of 5 superimposed responses. *a*, before application of solution; *b*, 2 min after application; *c*, 12 min after removal of solution and washing. The time scale shows 1 and 5 msec intervals. (A α spike retouched.)

tion for 15–20 min. Complete recovery of all the C fibres was even less frequent. Although 2 min application of the solution caused complete block of all these fibres, recovery followed washing with Ringer–Locke solution; longer applications than this caused irreversible changes in some of the C fibres. Thus it was found that the C fibres are the most susceptible to irreversible changes from the application of phenol in Myodil.

A 7·5% solution of phenol in Myodil. The effects of a 7·5% solution of phenol in Myodil on conduction in myelinated fibres of a posterior root

are shown in Fig. 4. The compound action potential showed components conducting at rates of 80, 24, 19, 16 and 14 m/sec. Two minutes after application of the solution the slowly conducting groups of fibres were entirely blocked, while the amplitude of the wave due to the fastest group of the A fibres had diminished to 25 % of its original height. The solution was applied for 3 min. Twelve minutes after removal of the solution and washing the roots the action potential of the fastest conducting group was 90 % of its original size, while that of the slowly conducting fibres showed slight recovery from blocking. Forty-five minutes after removal of the solution all fibre groups showed evidence of full recovery, except the most slowly conducting, of which the action potential was about 80 % of its original amplitude.

On comparing these rates of blocking of the fibres of the various groups, it was found that with a 7·5 % solution the rate was faster than with the 5 % solution. With regard to the recovery from the effects, the rate of recovery for the fastest conducting groups was not very different with the two concentrations, but in the more slowly conducting fibres recovery took longer after the stronger solution.

In an experiment with the 7·5 % solution in which the phenol solution was not washed off the roots but was permitted to remain in contact with them for more than 3 hr it was found that some recovery occurred in the conduction of the largest fibres, but none occurred in this time in the C fibres. In a further experiment with this concentration of phenol applied for 3 min there was no recovery in the C fibres within 70 min although some of the α fibres had recovered.

A 10 % *solution of phenol in Myodil* caused a rapid block of all fibre groups, A and C fibres, though the α group was not completely blocked by 3 min application of the solution. In two experiments in which the solution was left in contact with the root for 3 and for 3½ min no recovery occurred in the C fibres; the α action potential recovered to 2/3 of its original size.

A 1 % *solution of phenol in Myodil.* The effects of a 1 % solution on the α and γ fibres of an anterior root are illustrated in Fig. 5. After 20 min application, the spike height of the γ group was very much reduced, while that of the α group was diminished by only about 10 %. The solution was removed after 24 min. The action potentials of both groups returned to their original heights, after removal of the solution and washing. This however did not invariably occur with the 1 % solution; in some experiments with this concentration there was a permanent fall in the height of the γ action potential.

A 0·5 % *solution of phenol in Myodil.* An experiment with the procedure described under Methods (p. 567) on the effects of a 0·5 %

solution of phenol in Myodil on the A and the C fibres of a posterior root is illustrated in Fig. 6. This solution was applied to the root for 15 min. At the end of this time the size of the action potential of the A fibres conducting at 80 m/sec had diminished in height by 10%. The solution was drawn off after 15 min application, and the root washed with Ringer–Locke solution. This root was then set up as a C fibre preparation. At this time no C action potential could be obtained; a sample of this root removed before application of the phenol gave the C action potential illustrated in Fig. 6*c*. Thirty minutes later a trace of the C action potential

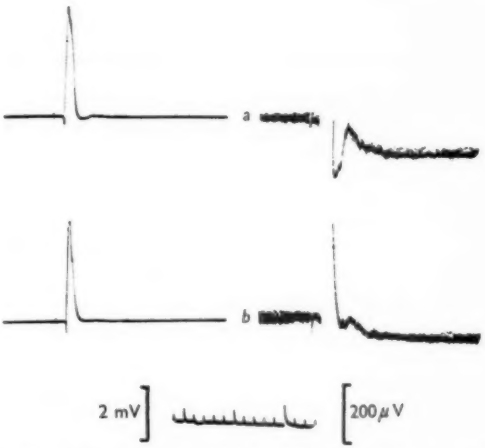

Fig. 5. Effect of 1% solution of phenol in Myodil on conduction in myelinated fibres of an anterior root. Each record consists of 5 superimposed responses. *a*, before application of solution; *b*, 20 min after application. The time scale shows 1 and 5 msec intervals. (Aα spike retouched.)

could be seen, and after a further 20 min a C action potential was obtained. Thus a weak solution of phenol in Myodil blocks all the C fibres of a root and during this period of application it blocks only a few of the largest A fibres. A 0·5% solution, however, does not permanently block all C fibres when it is applied for 15 min.

Phenol in aqueous solution

It was assumed in this work that there would be aqueous concentrations of phenol having equivalent effects on conduction to certain concentrations of phenol in Myodil. Experiments were undertaken to demonstrate the effect of weak solutions of phenol dissolved in Ringer–Locke solution.

The effects of a 0·5 % aqueous solution of phenol on α fibres is illustrated in Fig. 7. It will be seen that 180 sec after the application of the solution the height of the α wave of the A group has been reduced by 80 %. After 3½ min the solution was removed and the root was washed with Ringer–Locke solution. Twenty-six minutes later the α wave had recovered to 40 % of its original height. A weaker solution of phenol, 0·1 % in Ringer–Locke solution, reduced the height of the α wave by 20 % in 3 min; after removal and washing complete recovery was obtained.

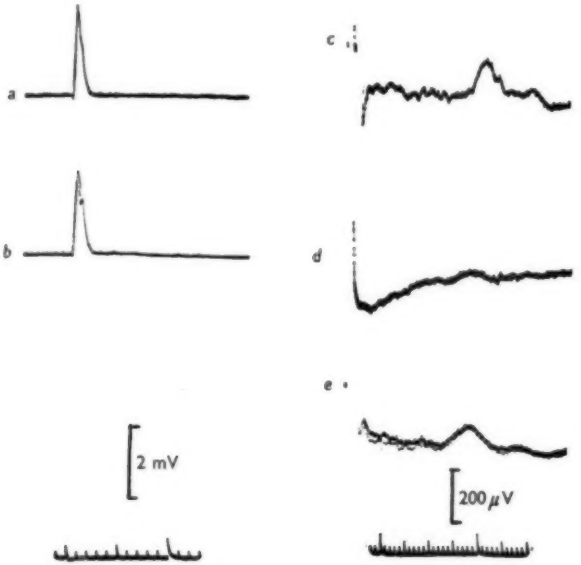

Fig. 6. Effect of 0·5 % solution of phenol in Myodil on conduction in myelinated and C fibres of a posterior root. Each record consists of 5 superimposed responses. *a*, A fibres before application of solution; *b*, 15 min after application; *c*, C action potential in control root; *d*, C action potential of treated root 30 min after removal of solution and washing; *e*, 50 min after removal of solution and washing. The time scales show 1, 5; and 20 msec intervals. (Aα spike retouched.)

The effects of a 0·5 and 0·1 % aqueous solution on the C fibres is shown in Fig. 8. The solutions were applied for 5 min each. The records used for illustration were selected to show the effects at comparable times. The 0·5 % solution caused diminution in the size of the action potential immediately the solution was applied. After 10 sec the C action potential was almost entirely removed, whereas at this time the 0·1 % solution had only a slight effect. After 35 sec application of the 0·1 % solution there was a great diminution in the size of the action potential. No C action potential was

seen at 2 min 45 sec. The solutions were removed after 5 min, and the roots washed. Forty-five minutes after removal the C action potentials had returned, though they were not as large as before applying the solutions.

The effects may now be compared of phenol in aqueous and in Myodil solutions under these experimental conditions. With regard to the effect on C fibres the experiments illustrated in Figs. 3 and 8 can be compared.

Fig. 7

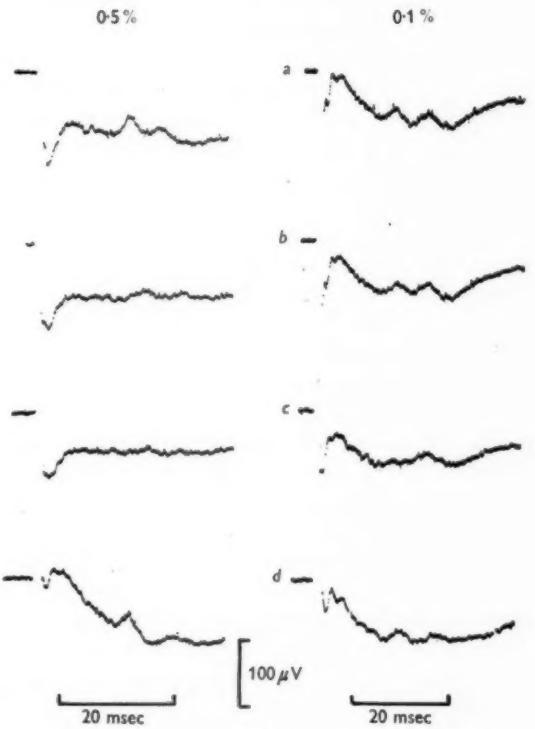

Fig. 8

Fig. 7. Effect of 0·5 % phenol in Ringer–Locke solution on conduction in myelinated fibres of a posterior root. Each record consists of 5 superimposed responses. *a*, before application of solution; *b*, *c*, *d*, 15, 90 and 180 sec after application; *e*, 26 min after removal of solution and washing. The time scale shows 1 msec intervals.

Fig. 8. Effect of 0·5 and 0·1 % phenol in Ringer–Locke solution on conduction in C fibres of a posterior root. *a*, before application of solution; *b*, *c*, 10 and 35 sec after application; *d*, 45 min after removal of solution and washing.

With 0·1 % phenol in Ringer–Locke solution there was a marked reduction of the action potential in 35 sec. A similar reduction was achieved with a 5 % solution of phenol in Myodil between 20 and 85 sec. In fact, when the heights of these C action potentials were measured in the actual experiments illustrated it was found that they were reduced to half their original height in about 30 sec in both cases. When a 7·5 % solution of phenol in Myodil is used the C action potential is completely blocked within 30 sec. The results of other experiments support this comparison, that a 5 % solution of phenol in Myodil is roughly equivalent to a 0·1 % aqueous solution.

Comparing the effects of stronger aqueous solutions with those of 7·5 and 10 % phenol in Myodil, blocking of the C fibres was found to be complete in 10 sec with 0·5 % aqueous solution; 10 sec after application of 7·5 or 10 % solution of phenol in Myodil, however, the C action potential is still large. Similar results from other experiments support the conclusion that 0·5 % aqueous solution is more potent than a 7·5 % or 10 % solution of phenol in Myodil.

Procaine solution

As it was found that phenol either in Myodil or in aqueous solution blocks nerve conduction, that its action is reversible, and that its effect falls more heavily on the C fibres than on large myelinated fibres of the root, it seemed advisable to compare its action with that of a standard local anaesthetic under the same conditions. Procaine was chosen for this comparison.

The effects of 0·2 % procaine in Ringer–Locke solution on the α and γ fibres of the A group of an anterior root are illustrated in Fig. 9. One minute after application the height of the α wave had fallen by less than 5 %, whereas that of the γ wave had fallen to 1/3 of its original height. Eleven minutes after the application, the height of the α wave had fallen by 30 %, and the γ wave had disappeared. Twenty-three minutes after removal of the solution and washing, the α wave had returned to within 5 % of its original height, but the γ wave was still absent. One hour after removal of the solution, the heights of both action potentials had returned to their original values.

On comparing this result with those in which phenol in Myodil was used, it was found that a 5 % solution of phenol in Myodil was far more effective in blocking all groups of fibres than the 0·2 % solution of procaine. In most of the thirty experiments the action potential of all fibres of the A group had ceased to be measurable after 4–5 min of application of 5 % phenol in Myodil. To obtain this result with procaine it was found that a 0·5 % solution had to be used.

With regard to a comparison of phenol in Myodil and procaine on the C fibres, the effect of a 0·1 % solution of procaine is shown in Fig. 10. This concentration exerts an immediate effect on the action potential of these fibres; after 15 sec, only a trace of the action potential can be seen, and by 30 sec it is completely abolished.

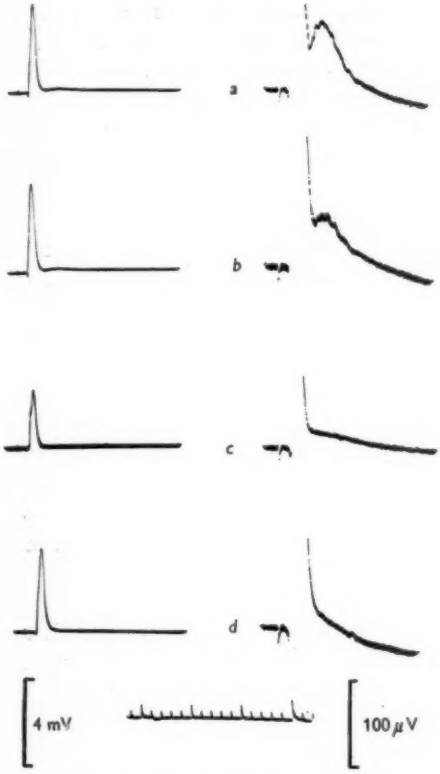

Fig. 9

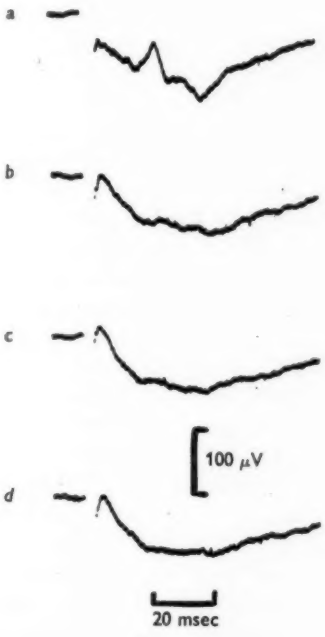

Fig. 10

Fig. 9. Effect of 0·2 % procaine in Ringer–Locke solution on conduction in fibres of an anterior root. Left column at low amplification to show fast conducting fibres (α range), right column at high amplification to show slowly conducting fibres (γ range). Each record consists of 4 superimposed responses. *a*, before application of solution; *b*, *c*, 1 and 11 min after application; *d*, 23 min after removal of solution and washing. The time scale shows 1 and 5 msec intervals. (Aα spike retouched.)

Fig. 10. Effect of 0·1 % procaine in Ringer–Locke solution on conduction in C fibres of a posterior root. *a*, before application; *b*, *c*, *d*, 15, 25 and 30 sec after application.

In comparison, a 5 % solution of phenol in Myodil took 45 sec to block the action potential, and an aqueous 0·1 % solution of phenol took 35 sec. A stronger, 0·5 %, solution of procaine removed the C action potential as soon as it was placed around the root.

DISCUSSION

It has been shown that phenol blocks conduction in nerve fibres, whether it is dissolved in Myodil, glycerol, or water. The blocking is somewhat selective in that it affects certain groups of fibres more than others. This selective action of phenol was observed by Iggo & Walsh (1959). The rate and the amount of blocking and of recovery are dependent on the concentration of phenol in the solution. The action is the same whether it is dissolved in water, in Myodil, or in glycerol. As dilute solutions of aqueous phenol are as active as concentrated solutions of phenol in Myodil or glycerol, and as the solvents themselves have no effect on conduction, it seems reasonable to assume that to affect conduction the phenol surrounding the nerve fibres must be in aqueous solution. The effect of the solvent is probably to determine the rate of liberation of the phenol.

The blocking of conduction is reversible or irreversible, depending on various factors such as the length of time the solution is allowed to act, the concentration of phenol in the solution, the solvent used for the phenol and the group of fibres involved. When the solution of phenol is placed in contact with a root for a certain time, it can cause irreversible block of the C fibres and a reversible block of the α fibres. Even a short time in an adequately concentrated solution of phenol produces an irreversible block of the C fibres.

The experiments reported here throw light on the early changes that occur when phenol in Myodil is used for the treatment of intractable pain in man. When a 7·5 % solution is injected intrathecally, there is a period of local anaesthesia and paralysis coming on within 50 sec of the injection; this is followed by a gradual recovery, taking about 20 min. This local anaesthesia and paralysis may be correlated with the reversible block of conduction shown in these experiments.

These solutions of phenol not only have a temporary action, but some permanent damage to nerve fibres is caused. Chronic experiments on the long-term effects of phenol are still under way; until they are finished, it is impossible to correlate the chronic effects found in man with the experimental findings in the cat.

With regard to the action of phenol as a local anaesthetic, it was never found to stimulate nerve fibres. Before causing complete block, phenol caused Wedensky inhibition, the fibres failing to conduct trains of im-

pulses. Phenol blocks the various fibre groups of a nerve root in the same order as do standard local anaesthetics; C fibres are blocked first, and the fastest conducting A fibres are blocked last. Phenol in Myodil and in aqueous solution, in appropriate concentrations, thus acts similarly to recognized local anaesthetics; and so it seems proper to class it as such, as some pharmacologists do.

In experiments on amoebae, Goldacre (1952) has shown that phenol, in common with many lipid-soluble substances, renders these organisms insensitive to touch. He also showed that the reversibility of this change was related to the duration of application and the concentration of the phenol in the culture fluid. He was able to vary these factors so that the amoebae either suffered no permanent effect from the temporary anaesthesia or they were killed.

We have observed irreversible blocking lasting for the duration of our experiments. This may have been due to a prolonged local anaesthetic effect; for the longest period of observation following application of the solution was 3 hr, and at this time it is possible that the fibres might still have been only temporarily blocked. On the other hand, it is probable that the failure to recover from blocking at 3 hr or less may indicate a true irreversible change in the affected fibres. That histological changes can occur with concentrations of phenol in Myodil of 5 % has been reported by Nathan *et al.* (1958).

It could be objected that since phenol damages nervous tissue it should not be classed as a local anaesthetic. Yet this objection should apply to other local anaesthetics and even to those most commonly used. It certainly applies to cocaine, the first local anaesthetic introduced; and it had been shown by Lundy, Essex & Kernohan (1933) that procaine, which has been found to be very useful on account of its high safety margin, can cause permanent damage to the nerve fibres of the spinal cord when it is used in a sufficiently high concentration. Phenol is different in that it has a low safety margin; for it is probably impossible to produce a block of all the fibres of a root without causing damage to some of them. It is fortunate that phenol has both temporary and permanent blocking effects, for this permits it to destroy nerve fibres, having blocked their conduction; thus it achieves their destruction without causing pain.

SUMMARY

1. An investigation is reported of the effects of phenol dissolved in ethyl iodophenylundecylate (Myodil) and in Ringer–Locke solution on nervous conduction in the spinal roots of the cat.

2. Phenol, according to its concentration, the length of time for which

it acts and the solvent in which it is dissolved, can cause a completely reversible or an irreversible block of nervous conduction in myelinated and unmyelinated nerve fibres.

3. The order in which the fibre groups are blocked is the same as that for local anaesthetics, small fibres before large fibres. The greatest differential action is seen between C and A fibres.

4. The irreversible blocking also affects the small fibres more than the large: recovery is most likely to occur in the fast conducting fibres of the A group and least likely to occur in the C fibres.

We take this opportunity of thanking Drs E. A. Carmichael and W. A. Cobb for facilities to do this work and for their help.

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THE MEASUREMENT OF METABOLIC AND VASCULAR RESPONSES IN LIVER AND MUSCLE WITH OBSERVATIONS ON THEIR RESPONSES TO INSULIN AND GLUCOSE

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The interpretation of temperatures recorded from most tissues of the body is a matter of some complexity. Four major factors may usually be considered as of primary importance, namely, the temperature of the blood flowing to the tissue in question, the rate of flow of blood in the tissue, the 'cooling power' of its environment and, lastly, the rate of metabolic heat production within the organ.

Thermocouples heated by means of a current passed through a small heating filament have recently been used by several workers to elucidate blood-flow change in internal organs. It might reasonably be assumed that metabolic heat production would have the same effect on a thermocouple thus artificially heated as on an unheated thermocouple. Acting on this assumption Grayson & Kinnear (1958) devised a method whereby blood flow could be estimated by means of a heated thermocouple technique (internal calorimetry, Grayson, 1952). Steps were taken to allow for the temperature of the blood flowing into the part and to allow for possible variations in heat loss from the part. The result was used to determine the unknown remaining variable, namely, metabolic heat production.

The purpose of the present paper is to report further investigations designed, in particular, to study the validity and scope of this approach to the measurement of metabolic heat production. The physiological aspects of the investigation are, therefore, not to be regarded as complete in themselves, for although interesting comparisons between liver and muscle have been revealed, the primary purpose of the observations was to demonstrate the usefulness of the method.

METHODS

Internal calorimetry

The technique of 'internal calorimetry' for the measurement of blood flow in internal organs has been fully described previously (Grayson, 1952). It depends on the thermoelectric measurement of thermal conductivity. It has been shown elsewhere that conductivity

increment (i.e. the thermal conductivity of living tissue less the thermal conductivity of dead tissue) is an approximately linear function of blood flow (Grayson, 1952).

There are at least three accepted methods for the evaluation of thermal conductivity with heated thermocouples. One is the method described by Grayson (1952) who used a manually operated apparatus and a 'cold junction' outside the organ under investigation. One is the method of Hensel, Ruef & Golenhofen (1954) which uses a needle-mounted heated thermocouple with the cold junction incorporated in the needle shaft. The other is the 'automatic' method of Carlyle & Grayson (1956), which also has a cold junction outside the organ. For reasons which will be apparent later this is the only method applicable to the determination of metabolic heat production.

This technique, in the form used in this laboratory, uses a simplified circuit (Grayson & Mendel, 1957) whereby current is supplied to the heaters of the thermocouple in cyclic fashion as follows: 0·200 A^2 for 24 sec; 0·600 A^2 for 17 sec; 0·315 A^2 for 24 sec; no current for 17 sec. This cycle is continuously repeated.

The heated thermocouple is of the type described by Grayson & Haigh (1954).

Cam-operated switch gear. The source of current is a 6 V car battery. A Muirhead decade resistance (10Ω) on one lead gives a rough control of input current. The other lead is attached to the common pole of three micro-switches operated by cams driven by reduction gear at a rate of one revolution/82 sec from a constant speed motor. There are four cam positions, so that each switch is brought into the circuit in turn, followed by a short period with all switches in the 'off' position.

This is a simplified version of previous circuits. There are no terminals. All connexions are within the box. The output leads pass straight to the heaters of the recording thermocouple. (The manually operated box described in previous papers has now been finally discarded.)

The recording apparatus consists of a Cambridge D'Arsonval galvanometer (internal resistance 50Ω) with a shaft of light recording on a slow moving recording camera (paper width, 120 mm). (In recent experiments a Cambridge four-channel recording camera with two channels converted to temperature recording, by substitution of a D'Arsonval unit for the standard oscillator, has been found satisfactory.)

Insertion of recorder. Wistar strain rats, average weight 200 g, were used in these experiments. Recorders were planted in the liver according to methods described previously (Grayson & Kinnear, 1958). The cold junction was situated intra-abdominally near the portal vein. In the present experiments it was found preferable to bring all leads to the surface of the animal and complete the constantan connexions of the thermo-electric circuit outside the animal.

In the present work all recording of liver blood flow and metabolism was made on the conscious animal. The leads from the apparatus were connected to the thermocouple leads and heater leads from the animal by soldering. The joints were covered with adhesive strapping and the animal was allowed free movement inside its cage.

For intravenous injections a polyethylene cannula was inserted into an external jugular vein at the same time as the insertion of the recorders, and brought out through the back of the neck. During an experiment a connecting length of polyethylene tubing enabled the animal to move freely in its cage, yet to receive intravenous injections without disturbance. Unfortunately it was not possible to withdraw sufficient blood through this type of cannula for blood-sugar estimations.

Muscle recording. In these experiments lightly anaesthetized rats were used. The recording thermocouple was inserted through a stab incision into the substance of the gastrocnemius muscle of one side.

For estimation of heat production it is necessary that the cold junction shall be in a situation representative of the temperature of the afferent blood. In some experiments the cold junction was accordingly introduced through the opposite femoral artery and pushed up to the aorta. In other experiments the cold junction was stitched into the mesentery near its

root (a much simpler and less traumatic procedure). Evidence will be adduced to demonstrate that under the conditions of these experiments mesenteric and aortic temperatures are linearly related. Consequently for most purposes it was thought sufficient to use the mesenteric situation for the cold junction. In all other respects the technique of recording from muscle was identical with that used for the liver.

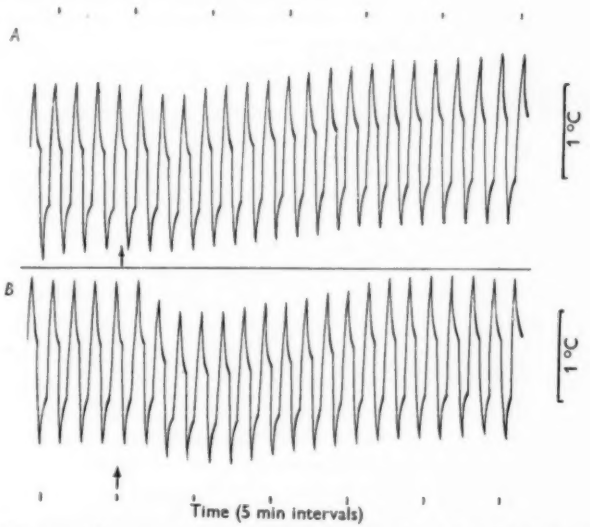

Fig. 1. Photographic records of liver temperature with a heated thermocouple in a conscious rat (tracing from original). *A*, intravenous insulin (2 u.) given at arrow. *B*, intravenous glucose (220 mg) given at arrow. Both records from same rat but on different days.

Determination of metabolic heat production

Figure 1 shows a typical record produced by the automatic recorder. The use of records of this type for the measurement of thermal conductivity and blood flow in living tissues has already been fully described. Nevertheless, the entire concept of metabolic heat production measurement depends on this beginning. Since the investigation of the validity of heat production measurement depends on an understanding of the physical principles involved, some recapitulation is probably necessary at this stage.

It has been shown (Grayson, 1952) that the temperature of a heated thermocouple embedded in a mass of tissue sufficient to be regarded as infinite is governed by the following relation

$$I^2R = 4\pi r\theta k$$

or, more simply, $I^2 = F\theta k$ (where F is an instrumental constant, I is the heating current, R the resistance of the heating elements, θ the temperature rise produced by the current and k the thermal conductivity of the substance or tissue in which the recorder is embedded). This relation applies strictly to a heated thermocouple through the heaters of which a steady current has been passed long enough to establish equilibrium. The present techniques employ a cyclic current in order to speed up temperature equilibration.

From the record shown in Fig. 1 it will be seen that when the current rises from 0 to 0·200 A^2, the temperature also rises to an 'equilibrium' level (lower equilibrium). When the

current rises to 0·600 A² (booster), the temperature rises suddenly to a peak; then when the current is lowered to 0·315 A² the temperature falls to another 'equilibrium' level (upper equilibrium). When the current is switched off altogether, the temperature falls below lower equilibrium level.

The difference in temperature between upper and lower equilibrium levels forms the basis of measurement for the calculation of thermal conductivity. In the present work the actual level of the lower equilibrium forms the basis for the calculation of metabolic heat production.

Calculation of excess temperature. Starting from an arbitrary point on a record it is easy to measure the actual changes which occur in lower equilibrium temperature. It is also possible, starting from the same point, to determine the changes which should have occurred in lower equilibrium temperature if the only factor concerned were blood-flow change.

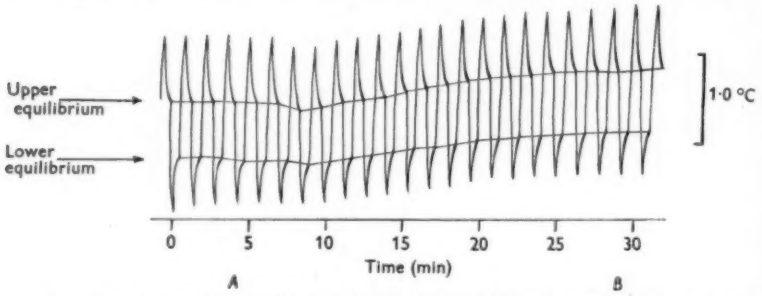

Fig. 2. Treatment of a recording in determination of excess temperature. Same tracing as Fig. 1*A*. Upper equilibria and lower equilibria joined. Vertical distance between upper and lower lines = θ. At *A* (before insulin), $\theta_1 = 0{\cdot}64°$ C; at *B* (after insulin), $\theta_2 = 0{\cdot}69°$ C. At *A*, temperature of lower equilibrium, t_1 measured from bottom of paper = 0·66° C; at *B*, lower equilibrium, $t_2 = 0{\cdot}96°$ C. Rise in lower equilibrium temperature = 0·3° C. Calculated temperature change due to blood flow alone = $\frac{200}{115}(\theta_2-\theta_1)$ (see text). Excess temperature

$$= (t_2-t_1)-\tfrac{200}{115}(\theta_2-\theta_1) = 0{\cdot}2°\text{ C}.$$

(In practice temperatures are measured in mm, conversions to °C being done by adjusting the ordinate of the derived graph of excess temperature.)

Measurement of actual change. Figure 2 (the same experiment shown in Fig. 1*A*) is a record of the effect of insulin on rat liver. The temperature change between point *A* and point *B* can be determined by measuring from the bottom of the paper at each point and subtracting. In Fig. 2 the lower equilibrium temperature rose by 0·3° C.

Prediction of change due to blood-flow change. The only assumption involved in the following calculation is that, at any one point on the record, I^2 and θ are linearly related.

Let θ be the difference between lower and upper equilibrium temperatures. In Fig. 2 at position *A*, $\theta = 0{\cdot}64°$ C. Under the conditions of thermal conductivity appertaining at that moment, this is the temperature rise produced by a current of 0·115 A^2.

But the lower equilibrium is maintained by a current of 0·200 A². By simple proportion, the temperature rise which would be produced by a current of

$$0{\cdot}200\text{ A}^2 = 0{\cdot}200/0{\cdot}115 \times 0{\cdot}64°\text{ C}.$$

At position *A* this = 1·1° C, which is the amount by which the heating current has raised liver temperature. At position *B*, where the conditions of thermal conductivity were different, the same calculation may be applied. At this position $\theta = 0{\cdot}69°$ C. Whence the amount by which the lower equilibrium heating current has raised liver temperature = 1·2°C. Subtracting the values obtained at *A* from those obtained at *B*, it can thus be estimated

that changes in thermal conductivity occurring as a result of alteration in blood flow should have produced a rise in lower equilibrium temperature of 0·1° C. This is 0·2° C less than what in fact occurred.

In the example shown in Fig. 1*B*, similar calculations show the opposite effect, the actual drop being greater than the calculated effect. Such temperature discrepancies between real and predicted changes will be referred to as 'excess temperature' and evidence will be adduced to demonstrate that 'excess temperature' is, in fact, due to changes in tissue metabolism.

RESULTS

Validity of blood flow and excess temperature calculations

By means of manual recording the validity of the relation, $I^2 = F\theta k$ has been amply demonstrated. For purposes of metabolic heat recording it is, however, necessary to ensure that the same relation holds good with automatic recording. For blood flow determination, I^2 being constant, it will be sufficient to demonstrate linearity between $1/\theta$ and k. For excess temperature determination, however, the relation between $1/\theta$ and k does not enter into the calculation and it will be sufficient to demonstrate linearity between I^2 and $1/\theta$.

Relation between $1/\theta$ *and* k. For the purpose of blood-flow measurement this relation has probably been sufficiently confirmed by previous work. Thus, using a wide series of gelatine/water and gelatine/water/graphite gels, Carlyle & Grayson (1956) showed a linear relation between $1/\theta$ as obtained by the photographic method and thermal conductivity measured by the manually controlled heated thermocouple technique (Grayson, 1952). Further, perfusion experiments in the brain also showed linearity between $1/\theta$ and blood flow.

Later in this paper, however, calculations will be presented which depend on the full validity of the equation, $I^2 = F\theta k$. It was thought desirable, therefore, to repeat these experiments. Figure 3*B* shows the relation between $1/\theta$, recorded photographically, and k obtained by the method of Grayson (1952), using for the purpose a series of gelatine/H_2O and gelatine/graphite/H_2O gels. It is apparent that the photographic method can be used with complete accuracy for the determination of absolute thermal conductivity. Figure 3*A* shows the results of a typical perfusion experiment in which the liver was perfused with dextran solution through the portal vein. The inflow was controlled and measured. It will be seen that the relation between $1/\theta$ and flow was completely linear over the range of perfusion rates selected.

Relation between I^2 *and* θ. Linearity between I^2 and θ has been demonstrated with thermocouples heated by a simple heating current (Grayson, 1952). It has never been confirmed with automatic recording. Since all calculations are limited to upper and lower equilibria it will be sufficient to demonstrate linearity over the range from 0 to 0·315 A^2.

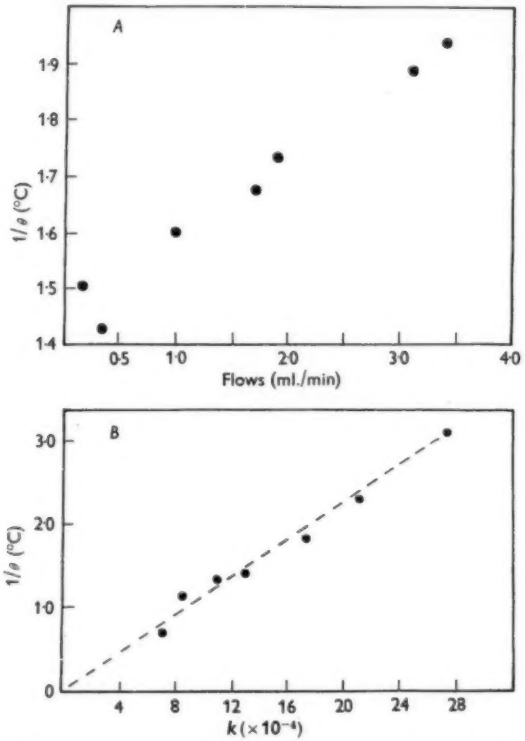

Fig. 3. *A*, Relation between flow and $1/\theta$; liver perfused with dextran through the portal vein. *B*, Relation between $1/\theta$ and thermal conductivity; readings from a series of gelatine/H_2O and gelatine/graphite/H_2O gels.

For this purpose the photographic records obtained during normal experiments afford sufficient proof on the basis of excess temperature calculations made at upper and lower equilibrium levels: Calculated on the assumption of linearity between I^2 and θ (see Methods), at lower equilibrium

$$\text{Excess temperature} = (t_2 - t_1) - \tfrac{200}{215}(\theta_2 - \theta_1).$$

Calculated in the same way at upper equilibrium

$$\text{Excess temperature} = (t'_2 - t'_1) - \tfrac{315}{115}(\theta_2 - \theta_1),$$

where t'_2 = upper equilibrium temperature at *B*,

t'_1 = upper equilibrium temperature at *A*.

But $t'_2 = (t_2 + \theta_2)$; $t'_1 = (t_1 + \theta_1)$,

Therefore excess temperature $= (t_2+\theta_2)-(t_1+\theta_1)-\frac{315}{115}(\theta_2-\theta_1)$

$= (t_2-t_1)+(\theta_2-\theta_1)-\frac{315}{115}(\theta_2-\theta_1)$

$= t_2-t_1-\frac{200}{115}(\theta_2-\theta_1).$

If the assumptions of linearity between I and θ were not correct the above relations could not hold, and different values of excess temperature would be obtained in upper and lower equilibria. In fact 300 random calculations have been made in twenty experiments. The results of four of the experiments are shown in Fig. 4. It will be seen that excess temperature was always the same whether calculated from upper or from lower equilibrium levels.

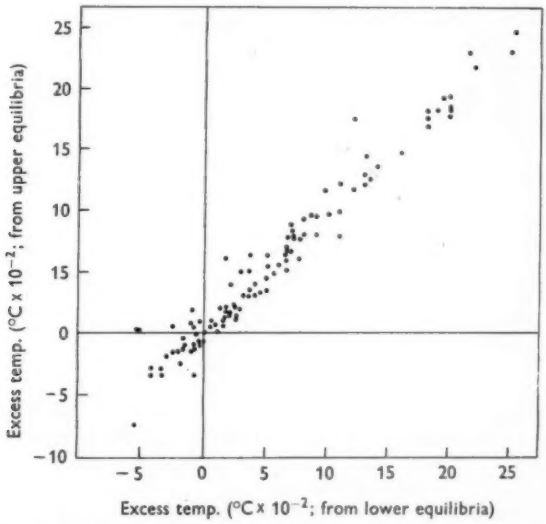

Fig. 4. Relation between I^2 and θ. Excess temperature from four different experiments; ordinate, calculations from upper equilibrium; abscissa, calculations from lower equilibrium.

It must, therefore, be concluded, that under the conditions of the present experiments, I^2 and θ are linearly related.

Experimental assumptions. Reference has already been made to these as regards the liver (Grayson & Kinnear, 1958). There are two. One is that the 'cold junction', fixed to the mesentery near the portal vein, represents closely the mean temperature of the blood flowing to the liver. The other is that heat loss from the liver through the diaphragm and body wall is a fairly constant factor reacting only slowly on over-all liver temperature. The experimental justification of these assumptions has already been fully reported.

With respect to the muscle, for many experiments the cold junction was inserted through a femoral artery into the aorta near its bifurcation. It is reasonable to suppose that this situation represents the temperature of the blood flowing to the limb. In other experiments the cold junction was stitched to the root of the mesentery near the aorta. Figure 5 shows the results of an experiment in which simple temperature was recorded from the aorta and the mesentery during an insulin experiment. It will

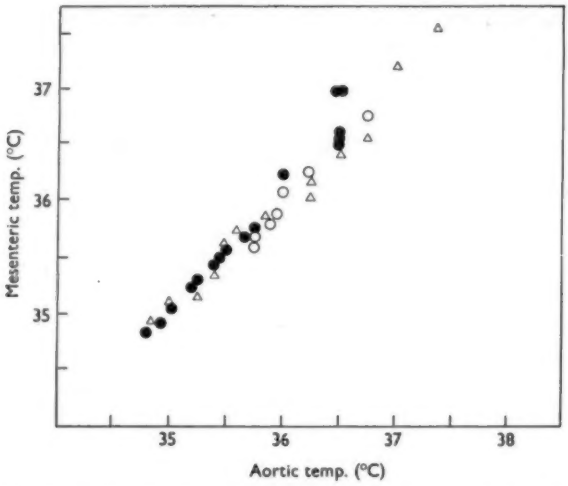

Fig. 5. Relation between the temperature of the mesentery and that of the aorta in three rats. Temperature changes induced by means of pentobarbitone and insulin.

be seen that both temperatures reacted similarly. Similar results were obtained in all such experiments and it was considered justifiable to use the mesenteric situation for the cold junction. Variations in heat loss are not considered to be such a likely source of error as in the liver since the limb was always wrapped in cotton-wool, and the experiments carried out under conditions of constant temperature.

Liver blood flow and metabolism

Conscious rats were used in experiments to determine the effects of insulin, glucose and combined insulin and glucose, on liver blood flow and temperature. After the insertion, under light ether anaesthesia, of a blood-flow recorder in the liver, a cold junction in the mesentery and a polyethylene cannula into a jugular vein, the animals were allowed to recover consciousness before recording was begun. Occasional restless

animals were given 0·02 ml. pentobarbitone sodium through the polyethylene cannula to produce mild sedation.

Insulin. The effect of insulin on liver blood flow and temperature was determined in six experiments. After a preliminary period of recording for 30 min, insulin (Insulin A.B., British Drug Houses, Ltd., 40 u./ml.;

TABLE 1. The effect of insulin on liver blood flow and calculated excess temperature. Values for liver blood flow are expressed as thermal conductivity increments ($\delta k \times 10^{-4}$) above the thermal conductivity of dead liver

	Blood flow		Metabolism, maximum change in excess temperature ($°C \times 10^{-2}$)
Expt. no.	Average initial δk	Average maximal δk after insulin	
1	6·8	9·4	+13
2	5·3	7·5	+12·5
3	1·5	2·3	+19
4	7·8	12·6	+ 6
5	7·4	6·3	+ 6
6	0·6	1·2	+18

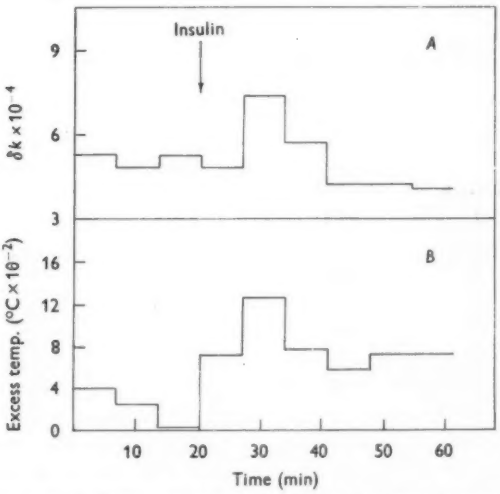

Fig. 6. The typical effect of insulin (I.V.), *A*, on blood flow (δk); *B*, on metabolic heat production (excess temperature) in the liver of a conscious rat.

1 i.u./100 g rat) was given intravenously and recording continued for a further 60 min. The changes in the thermal conductivity of the liver (expressed as thermal conductivity above that of the dead liver (δk) and the calculated 'excess' temperature are shown in Table 1. The pattern of a typical response is shown in Fig. 6.

It can be seen that in three experiments there was a rise in δk values amounting to 38–62 % of the initial values. In one experiment there was a slight fall in δk values, and in two experiments, where the initial δk values were low, being $1{\cdot}5 \times 10^{-4}$ and $0{\cdot}6 \times 10^{-4}$, there was probably no change. In the experiments in which a rise in δk values occurred the change started within 10 min after giving insulin, and was maintained for a further 10–30 min. The response shown in Fig. 6*A* represents Expt. 2 (Table 1), and the blocks are the average δk values for five successive cycles.

The results for the calculated maximum excess temperature were more constant than the blood-flow response, a rise occurring in every case (Table 1). The maximum calculated excess temperature varied from 6×10^{-2} to 19×10^{-2} ° C. The response shown in Fig. 6*B* has also been plotted from the average calculated values in five successive cycles. The rise in excess temperature started within 10 min of insulin injection, reached a maximum in from 10 to 20 min, and then fell. In many experiments, however, it remained above resting values for the remainder of the experiment.

TABLE 2. The effect of glucose on liver blood flow and calculated excess temperature

	Blood flow		
Expt. no.	Average initial δk	Average maximal δk after glucose	Metabolism, maximum change in excess temperature (°C × 10^{-2})
1	6·0	8·4	−29
2	3·3	7·1	−17
3	3·3	4·3	−20
4	6·3	8·3	+20
5	4·7	6·8	+13
6	3·7	5·0	+30
7	8·9	10·4	−12
8	1·0	2·1	−48
9	11·8	12·2	−20
10	5·9	7·4	−65

Glucose. The effect of glucose on liver blood flow and metabolism was determined in ten experiments. Glucose was given through the polyethylene cannula in the dose 100 mg/100 g rat, 100 mg glucose being dissolved in 0·3 ml. saline. The change in δk values and calculated excess temperature are shown in Table 2, and a typical response (Expt. 2) is shown in Fig. 7.

It can be seen from Table 2 that there was an increase in δk values in eight experiments amounting to from 17 to 100 % of the initial values. In one experiment (Expt. 9) there was no significant change in flow, and in Expt. no. 8, where the initial δk value was $1{\cdot}0 \times 10^{-4}$, the change was

probably also not significant. Changes in δk values started within 10 min of giving glucose, and in five experiments reached a maximum within 20 min, and then declined to resting values after a further 10 min. In three experiments, however, the rise in δk values occurred more slowly and remained high for the rest of the experiment (Fig. 7*A*).

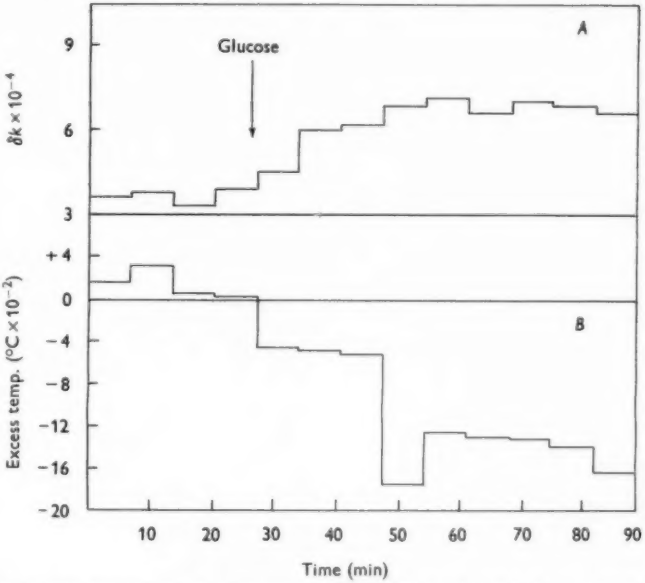

Fig. 7. The typical effect of glucose (100 mg/100 g rat, I.V.) *A*, on blood flow; *B*, on metabolic heat production in the liver.

In seven of these experiments there was a fall in the calculated average excess temperature (Table 2) varying from -12 to -65×10^{-2} °C. In three experiments there was a rise in excess temperature. The fall in excess temperature occurred within 5 min of the injection of glucose, the temperature remaining depressed for over 60 min in four experiments (Fig. 7*B*), and for 30 min in three experiments.

Glucose and insulin. The effect of combined glucose (100 mg/100 g rat) and insulin (1 i.u./100 g rat) on liver blood flow and temperature was determined in ten experiments. The results for δk values and excess temperature are shown in Table 3, and the varying pattern of response in Fig. 8. From Table 3 it can be seen that there was an increase in δk values in six experiments, a fall in two and no change in two experiments. In the experiments in which there was a rise in δk values the response, which was usually slight, occurred within 5 min and lasted from 10 to

20 min (Fig. 8*B*). Where a fall in δk values occurred, the response tended to persist for some 50 min before recovery to initial δk levels.

The changes in calculated excess temperature shown in Table 3 were very variable. In five experiments there was a rise in calculated excess temperature, in two of which the changes were only 1 and 4×10^{-2} ° C.

Figure 8 shows the pattern of response in three experiments. In the experiment shown in Fig. 8*A* there was no change in flow, and a small rise in excess temperature occurred 30 min after injection of glucose and insulin. In the experiment shown in Fig. 8*B* there was a rise in blood flow

TABLE 3. The effect of glucose and insulin on liver blood flow and calculated excess temperature

Expt. no.	Blood flow: Average initial δk	Blood flow: Average maximum δk after glucose and insulin	Metabolism, maximum change in excess temperature (°C × 10^{-2})
1	6·0	7·2	− 4
2	3·2	3·8	+ 4
3	5·6	4·4	+ 5
4	3·0	1·7	−19
5	1·0	3·0	+ 1
6	1·3	2·9	+12
7	4·6	6·6	−23
8	16·7	16·5	−50
9	8·4	11·4	+14
10	3·2	4·3	− 3

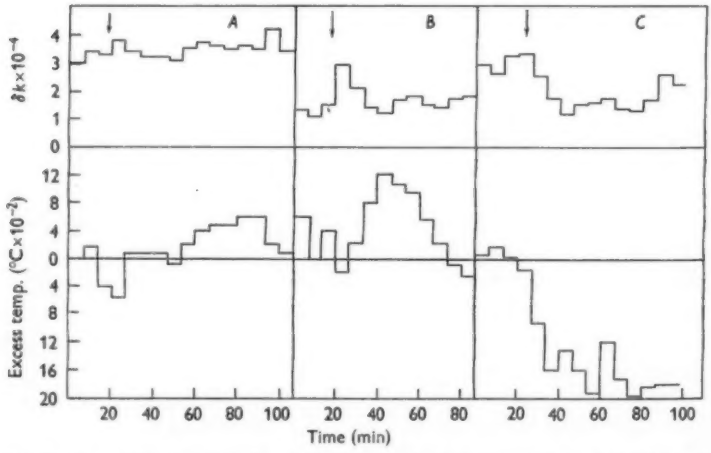

Fig. 8. Liver blood flow and metabolism; typical responses to combinations of insulin (1 i.u./100 g rat) and glucose (100 mg/100 g rat; three experiments *A*, *B* and *C*). I.V. injections given at arrows.

of short duration and marked rise in calculated excess temperature, and in the experiment shown in Fig. 8*C* there was a fall in blood flow which persisted for 60 min, and a marked fall in calculated excess temperature.

The effect of adrenalectomy. In nine experiments the adrenal glands were removed at the time of the insertion of the liver blood-flow recorder and the cold junction, and the experiments with insulin, glucose and glucose and insulin were repeated 2 hr after operation.

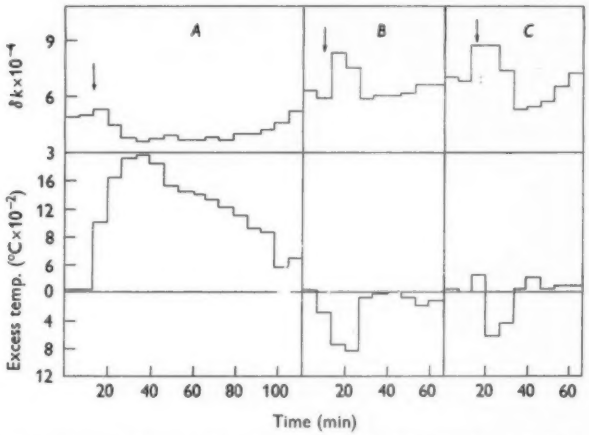

Fig. 9. The influence of acute adrenalectomy on blood flow and metabolic responses of rat liver, *A*, to insulin alone; *B*, to glucose alone; *C*, to a combination of glucose and insulin. All doses as before; I.V. injections given at arrows.

The results are shown in Fig. 9. Figure 9*A* shows a response with insulin alone. In this experiment there was a fall in δk values and a rise in calculated excess temperature. A fall in δk values occurred in two of the three experiments and in the other there was no change. A rise in calculated excess temperature occurred in all these experiments. Figure 9*B* shows the response with glucose. It can be seen that there was a rise in δk values of short duration, and a moderate fall in calculated excess temperature. A similar response was obtained with glucose and insulin (Fig. 9*C*). These changes were obtained with the other two experiments in each group.

Muscle blood flow and metabolism

Records were obtained from the belly of the left gastrocnemius muscle of the lightly anaesthetized rat (0·15 ml./100 g pentobarbitone sodium, reinforced in some cases by one further dose of 0·05 ml.). Intravenous injections were made through a cannula in the external jugular vein. In

each case an initial period of 1 hr was allowed with the heater circuits running for equilibration before recording was begun.

The effect of insulin on muscle blood flow and metabolism. The results of a typical experiment are shown in Fig. 10. Insulin (2 u.) was injected after recording the base line for 20 min. No significant change occurred in blood flow. There was a marked increase in excess temperature, which

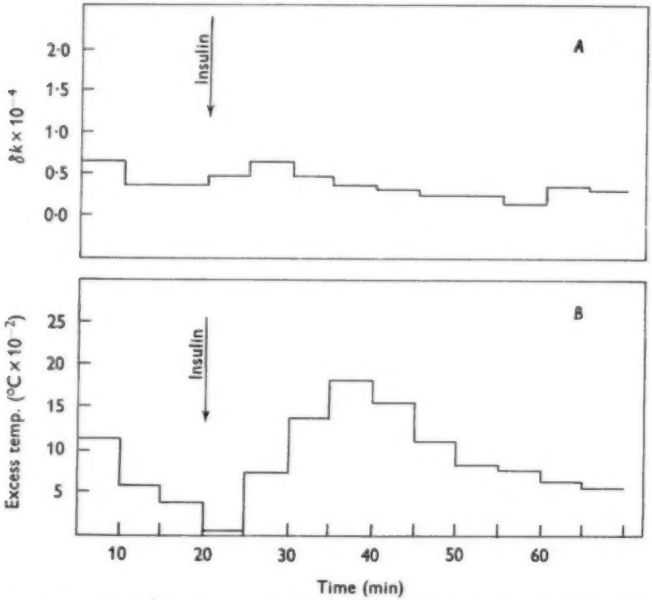

Fig. 10. Effect of insulin *A*, on blood flow (δk), and *B*, on metabolic heat production (excess temperature) in rat gastrocnemius muscle.

reached a maximum 20 min after giving insulin. Thereafter excess temperature subsided slowly to resting levels. Table 4 gives the results of eleven similar experiments. It will be seen that significant changes in blood flow were recorded in only three experiments, but that excess temperature rose in nine, the peak effect occurring 20–40 min after the injection.

The effect of glucose on muscle blood flow and metabolism. Figure 11 shows the results of a typical experiment in which 220 mg glucose was injected intravenously after 20 min of base-line recording. There was no change in blood flow. Excess temperature, however, fell markedly, reaching its lowest point 40 min after the injection. Thereafter it began to recover slowly.

Table 5 gives the results of eleven similar experiments. The effects on blood flow were usually slight. In three instances a slight rise was recorded,

in one case there was a fall, in the others no significant blood-flow change could be detected. The effects on excess temperature were more pronounced. In nine experiments there occurred pronounced depressions in excess temperature and metabolic heat production. In two experiments small

TABLE 4. The effect of I.V. insulin on rat skeletal muscle blood flow and excess temperature

Expt. no.	Change in δk	Maximum change in excess temperature ($°C \times 10^{-3}$)	Time to maximum effect (min)
1	nil	nil	—
2	nil	nil	—
3	nil	+2·5	35
4	nil	+1·1	20
5	0·6 rising to 1·2	+4·4	20
6	nil	+0·2	40
7	nil	+4·1	40
8	1·9 rising to 5·9	+4·9	40
9	4·7 rising to 5·2	+4·4	40
10	nil	+4·3	20
11	nil	+4·5	30

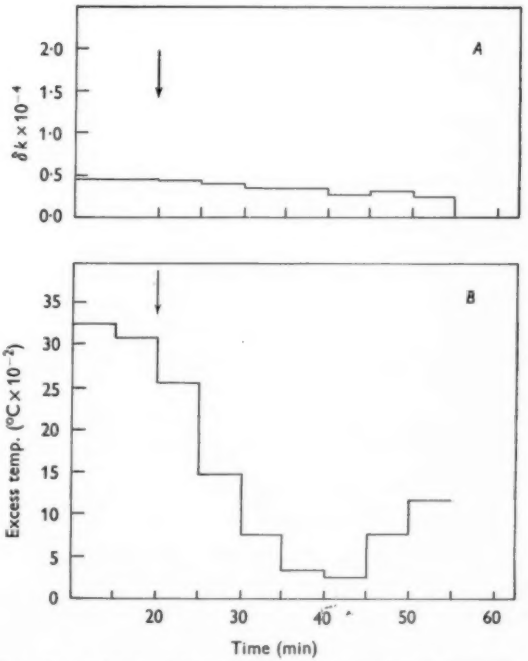

Fig. 11. Effect of I.V. glucose (100 mg/100 g rat), *A*, on blood flow; *B*, on metabolic heat production in rat gastrocnemius muscle.

TABLE 5. The effect of I.V. glucose on muscle blood flow and excess temperature

Expt. no.	Change in δk	Maximum change in excess temperature (° C × 10^{-2})	Time to maximum effect (min)
1	nil	−2·5	17
2	nil	−16·4	50
3	1·8 rising to 3·1	−3·7	10
4	nil	−1·8	50
5	3·7 rising to 4·8	−1·4	50
6	8·2 rising to 10·8	−0·5	50
7	nil	−0·6	45
8	nil	−0·4	10
9	4·2 falling to 1·3	+0·7	15
10	nil	+1·2	30
11	nil	−3·0	40

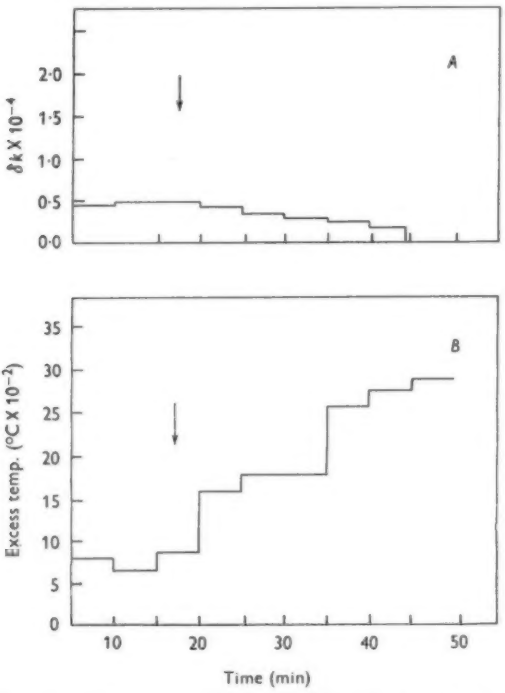

Fig. 12. Effect of insulin (1 i.u./100 g rat) and glucose (100 mg/100 g rat), *A*, on blood flow and *B*, on metabolism in rat gastrocnemius muscle.

increases were recorded. The timing of the maximum effects varied from 10 to 50 min after the injection.

The effect of glucose plus insulin on muscle blood flow and metabolism. Figure 12 shows the results of a typical experiment in which 2 u. insulin and 220 mg glucose were given together. No significant change occurred in muscle blood flow. There was, however, a marked effect on excess temperature which began to rise almost immediately reaching a plateau of effect after 20 min. The results for calculated excess temperature in six similar experiments are given in Table 6. In every case there was a pronounced rise in excess temperature beginning early and reaching a maximum in 20–45 min. There was no change in muscle blood flow in any of these experiments.

TABLE 6. The effect of I.V. glucose + insulin on rat skeletal muscle excess temperature

Expt. no.	Maximum change in excess temperature (°C × 10^{-2})	Time to max. effect (min)
1	+ 5·4	25
2	+13·9	45
3	+11·0	40
4	+ 8·4	30
5	+33·9	40
6	+21·2	20

Quantitative calculations of heat production

Excess temperature as used hitherto in this paper is merely a qualitative interpretation of changes in heat production beginning from an arbitrarily selected point. The following approach is an attempt to evaluate liver heat production in more absolute terms.

Heat changes between heated thermocouple and the surrounding tissues are governed by the equation $I^2 = F\theta k$. The fact that the liver itself is also a producer of heat is normally only indirectly allowed for. Consider two points A and B on the record (Fig. 2). Let the heat production of the liver at A be H_1 and at B, H_2. Let the temperature of the lower equilibrium, in excess of the afferent blood, at A be t_1 and at B, t_2. The square of the heating current at lower equilibrium = 0·200 A^2. Whence

(i) at A; $H_1 + 0{\cdot}200 = Ft_1k_1$ (t_1 and t_2 are herein substituted for θ in the formula $I^2 = F\theta k$);

(ii) and at B; $H_2 + 0{\cdot}200 = Ft_2k_2$.

But at position B, heat production in the liver has changed by an amount giving rise to an excess temperature e, which can be measured by methods already clarified. Let the difference between upper and lower equilibrium temperature be θ_1 at A and θ_2 at B.

At B, θ_2 is the temperature rise produced by 0·115 A²; whence a temperature of e would be produced by a current of $(0{\cdot}115e/\theta_2)$ A²

(iii) whence $$H_2 = H_1 + \frac{0{\cdot}115e}{\theta_2}$$

from (ii) $$H_1 + 0{\cdot}200 + \frac{0{\cdot}115e}{\theta_2} = Ft_2k_2,$$

but from (i) $$F = (H_1 + 0{\cdot}200)/t_1k_1;$$

(iv) whence $$H_1 + 0{\cdot}200 + \frac{0{\cdot}115e}{\theta_2} = \frac{t_2k_2}{t_1k_1}(H_1 + 0{\cdot}200).$$

In any experiment e, θ_2, k_1 and k_2 are known or can be calculated from the record; t_1 and t_2, however, give rise to difficulty. The value t_1 represents a temperature increment over and above the temperature of the inflowing blood produced partly by a heating current of 0·200 A², partly by the metabolic heat production of the tissue. In the case of liver there is considerable heat loss through the diaphragm and abdominal wall, which cannot be evaluated; t_1 cannot, therefore be measured directly. If the metabolic work done by the tissue were small in comparison to the work done by the heating element it might be sufficiently accurate to consider only the temperature increments produced by the heating current. In the case of liver these assumptions might not be justified.

The following calculation was made on a record from an experiment in which, although the blood flow changed, the recorded temperature difference between point A and point B did not change. The value t_2/t_1 was therefore unity and the calculation could be carried out without making any further assumptions. Figure 13 is the graph of excess temperature.

At position B, $e = 0{\cdot}23°$ C., $\theta_2 = 0{\cdot}71°$ C, $k_2 = 18{\cdot}8 \times 10^{-4}$, $k_1 = 16{\cdot}9 \times 10^{-4}$.

Applying the formula:

$$H_1 + 0{\cdot}200 + 0{\cdot}0374 = 1{\cdot}11H_1 + 0{\cdot}222,$$

whence $$H_1 = 0{\cdot}138 \text{ A}^2,$$

and $$H_2 = 0{\cdot}175 \text{ A}^2.$$

In two other experiments only were similar conditions found, i.e. no change in the level of t_1 and t_2, with, however, changes in k. In these, the corresponding values of H were 0·070 and 0·120 A², respectively. It is clear, then, that the heat production of the liver substance is very considerable and cannot be ignored in the calculation. At present, therefore, knowledge of heat production in the liver in quantitative terms is derived from these three rats. As will be shown, however, they do give some further information which may be of value.

The situation with regard to calculations in muscle is rather different. From three cases where the lower equilibrium temperature did not change, values for H were found to be 0·003; 0·005; 0·0004 A^2. These are exceedingly small and indicate a low resting metabolism, small in comparison with the heat produced by the heaters of the recorders. It is considered that in muscle, therefore, the tissue contribution to t_1 may be ignored and the

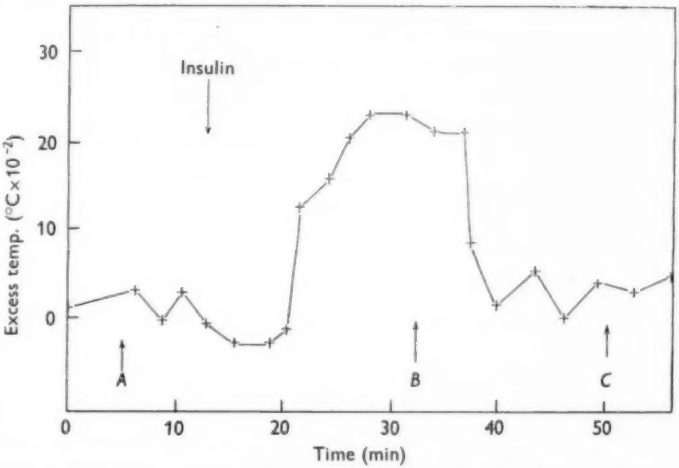

Fig. 13. Rat liver excess temperature changes following insulin; experiment to illustrate further calculations regarding heat production. In this experiment, although the blood flow changed, there was no change in lower equilibrium temperature between A and B, thus enabling heat production at A to be calculated in quantitative terms (see text). Values for position C were then determined from excess temperature and blood-flow data.

calculation applied more widely. The figures thus obtained give a comparison between heat production of the liver and the heat production of an electrically heated thermocouple operating under the same conditions in muscle.

It is, perhaps, difficult to visualize the meaning of this in terms of liver function. A further extension of the calculation, may, perhaps, clarify the issue. From the experimental values, at point A (Fig. 13), $\theta = 0{\cdot}77°$ C, i.e. a current of 0·115 A^2 produced a temperature rise of 0·77° C, whence a current of 0·138 A^2 would have produced a temperature rise of 0·92° C.

Now the flow of blood through liver is so high that the bulk of liver tissue available to be heated may be ignored. It may reasonably be assumed that most of the heat goes to raise the temperature of the blood flowing through the liver. Therefore 0·92° C is the temperature by which metabolic heat has raised that of the blood flowing into the liver. Now,

if the blood flow through the liver were known in absolute terms it would clearly be simple to calculate heat production in terms of calories. But although in the present experiments total flow has not been recorded, δk, which is a linear function of blood flow, has. Multiplying the metabolic temperature rise by δk will, therefore, give a figure in arbitrary units which is a simple function of heat production in calories.

Referring to the experiment above and applying this calculation to Fig. 13 we get:

at point A, $\delta k = 6{\cdot}2 \times 10^{-4}$; $\theta = 0{\cdot}77°$ C: metabolic temperature increment = $0{\cdot}92°$ C,

at point B, $\delta k = 8{\cdot}1 \times 10^{-4}$; $\theta = 0{\cdot}71°$ C: metabolic temperature increment = $1{\cdot}09°$ C,

at point C, $\delta k = 4{\cdot}6 \times 10^{-4}$; $\theta = 0{\cdot}83°$ C: metabolic temperature increment = $1{\cdot}09°$ C.

Whence, in arbitrary units, heat production is as follows:

$$\begin{aligned} \text{at point } A \quad & -0{\cdot}92 \times 6{\cdot}2 = 5{\cdot}7, \\ \text{at point } B \quad & -1{\cdot}09 \times 8{\cdot}1 = 8{\cdot}8, \\ \text{at point } C \quad & -1{\cdot}09 \times 4{\cdot}6 = 5{\cdot}6. \end{aligned}$$

For most of our present purposes this is as far as the calculations may be taken with any confidence. However, for the purposes of comparison with other data concerning liver metabolism the following approximation may have its interest.

In an earlier paper, Grayson (1952) suggested that a mean δk level of 12×10^{-4} usually approximated to an over-all liver blood flow of about 100 ml./100 g tissue/min. At point A, δk was $6{\cdot}1 \times 10^{-4}$. The average weight of the livers of the rats used in the present experiments was 8·0 g. It is reasonable, therefore, to assume that the total liver blood flow in this experiment was in the region of 4 ml./min. A further assumption which, in fact, is confirmed by unpublished observations on human liver (Grayson & Kinnear) is that no great temperature gradients occur within the liver. Now the temperature increment at point A (Fig. 13) produced by metabolic heat is $0{\cdot}77(0{\cdot}138/0{\cdot}115) = 0{\cdot}92°$ C. It is assumed that this temperature increment is evenly distributed. It follows, then, that in this rat the heat production of liver (this was a conscious rat) at rest was $0{\cdot}92 \times 4$ cal/min = 3·68 cal/min.

The calculation of heat production in terms of I^2 equivalents may, obviously, be equally applied to muscle. The further treatment of the figures may probably be applied with equal validity, since we are dealing only with fully equilibrated temperatures. At equilibrium the initial

task of heating the bulk of muscle tissue has already been accomplished. The further heat production is spent in heating the admittedly small flow of blood and in maintaining the temperature of the muscle bulk. In these experiments heat loss from the muscle was restricted by enclosing the limb in cotton-wool and may probably be ignored.

In calculations on muscle, however, consistency was difficult to obtain since the differences in total thermal conductivity before and after giving insulin were usually small and minor faults in recording made the value t_2k_2/t_1k_1 hard to evaluate reliably. The figures shown in Table 7 were from experiments which gave consistent results. A comparison of the figures from liver and muscle shows a very marked difference in resting heat production per unit mass of tissue, heat production in liver being almost 95 times that of resting muscle. On the other hand the effect of insulin is to raise the heat production of liver by 44%, that of muscle by 670%. Nevertheless, the gross rise produced by insulin remains far greater in liver than in muscle.

DISCUSSION

Measurement of metabolic heat production

The present paper presents a technique for the evaluation of metabolic heat production in liver and muscle. Most of the findings have been presented in terms of 'excess temperature', a simple concept indicating the difference between what actually occurs in liver or muscle and what would have occurred if, during the experiment, the only factor serving to alter tissue temperature were blood flow.

One assumption is of the highest importance, namely, that the cold junction is in a region representative of the temperature of the blood flowing to the organ. In the present work, this assumption presents little difficulty for reasons which have been fully described earlier in this paper, but it is apparent that instruments in which the cold junction is incorporated in the shaft of the needle housing the heated thermocouple (Hensel *et al.* 1954) would not be suitable for metabolic heat determinations.

The only other experimental factor which could easily affect the validity of the conclusions would be if large changes were to occur in the direct heat loss from liver or muscle. In the case of the liver it has been shown that, although the liver does, in fact, lose a considerable amount of heat by direct transfer through the diaphragm and through the abdominal wall, this factor cannot account for the phenomenon of 'excess temperature' (Grayson & Kinnear, 1958). In the case of muscle this factor is even less important since the experiments were carried out in constant-temperature rooms and the limb was, moreover, encased in cotton-wool so that the

ambient temperature of the limb approximated to that of the deep structures of the leg.

The only other major assumption is that I^2 and θ are linearly related. This point has been fully dealt with and there can be no doubt of the relation.

There can be little reasonable doubt that 'excess temperature' gives a qualitative picture of changes in metabolic heat production during the course of an acute experiment. The further mathematical treatment of the results, however, requires considerably more care.

In the first place the calculation cannot be applied to experiments where changes are too rapid. It is essential that reasonable time has been allowed for full equilibration of temperatures in relation to changed blood flow and heat production. In many experiments changes were too rapid for these conditions to be fulfilled. Accordingly, most of the results have been presented in the form of excess temperature and are intended to give merely a qualitative picture.

In a number of experiments (Table 7), however, consistent results were obtained. In these experiments it seems reasonable to suppose that the full calculation gives some indication of the quantitative changes, since the only assumption additional to those involved in 'excess temperature' is that blood flow and δk are linearly related. This has now been confirmed so many times on the basis of perfusion experiments that it can no longer be held in reasonable doubt (Grayson, 1952; Hensel *et al.* 1954).

Heat production in liver. Table 7 gives the resting heat production of liver in three rats. The figures are, of course, arbitrary units, the mean value being 3·4. On the basis of the approximations as to weight and blood flow dealt with earlier in this paper this is consistent with a mean heat production for the whole rat liver of 2·2 cal/min or 132 cal/hr. According to Behnke (1958) the total oxygen consumption of rat liver is 197 ml./hr, approximately 12 % of the rat's total consumption. This figure suggests a total heat output per hour for rat liver of approximately 98·5 cal.

Insulin and the liver

Blood flow responses. The present experiments add little to the findings of Grayson & Kinnear (1958) in this respect. The over-all response is confirmed, namely, that usually the hypoglycaemic phase is accompanied by a rise in liver blood flow.

The results of combining insulin with glucose were much the same as those previously reported. In most experiments a combination of these two substances produced no significant change in liver blood flow.

One must probably accept the conclusions of Grayson & Kinnear (1958) that insulin in doses sufficient to produce hypoglycaemia produces vasodilatation probably in the hepatic arterial radicles, that this vasodilatation is

not nervously mediated, nor can it be accounted for solely on the basis of adrenaline release.

Nevertheless, one additional fact has been elicited in the present work which further complicates the picture, namely, that glucose alone usually causes some significant increase in hepatic blood flow. It may be that this glucose vasodilatation has no connexion with the effects of insulin. The original suggestion of Grayson & Kinnear (1958), that insulin does not directly cause vasodilatation but produces its effect on blood flow only as a result of hypoglycaemia, may still be correct. Nevertheless, it is clear that the hepatic blood-flow responses in relation to blood-sugar levels are more complex than had been thought and that the present evidence is insufficient to warrant further speculation at this stage.

TABLE 7. Liver and muscle heat production per unit mass of tissue (arbitrary units).

	Liver		Muscle	
	Pre-insulin	Post-insulin	Pre-insulin	Post-insulin
	5·64	8·82	0·011	0·15
	2·42	2·90	0·020	0·18
			0·115	0·85
			0·030	0·15
	2·10	3·10	0·005	0·06
Means	3·4	4·9	0·036	0·28

Heat production responses. The heat production responses in the liver were more consistent in many ways than the blood-flow changes. In the first place insulin always produced a rise in excess temperature. The extent of the rise was variable, but from Table 7 it is seen that the mean changes were from a resting heat production of 3·4 arbitrary units per unit mass of tissue to 4·9 units per unit mass, i.e. a mean rise in heat production of 44 %.

The effect of glucose alone was not so constant but in seven out of ten experiments there was a definite fall in excess temperature.

The effect of combining insulin and glucose was to produce a variable picture. In five experiments there was a fall; in five there was a rise. But in most cases the changes were small and the general picture of reaction was of a response intermediate between that produced by insulin alone and that produced by glucose alone. Clearly, measurements of blood glucose levels would have helped greatly in assessing the results; unfortunately technical considerations made it impossible in these experiments.

The evidence at present does not permit a definitive conclusion as to the origin of the increased heat production that follows administration of insulin. The fact that glucose alone usually depresses heat production and

that a combination of insulin and glucose radically modifies the insulin effect suggests that it may be the level of blood glucose rather than the direct effect of insulin itself which determines the reaction. If this be so it is hard to avoid the further conclusion that it is the break-down of liver glycogen (in response to the low blood sugar) that is the main exothermic reaction involved.

This argument may be taken somewhat further. One of the effects of imposing on the animal a raised blood sugar by means of intravenous injection of glucose might be expected to be the inhibition of glycogen break-down. This procedure usually also inhibited heat production, a finding which adds further weight to the possible importance of glycogen break-down as the exothermic reaction concerned.

Muscle and insulin

Blood-flow responses. It has been reported elsewhere that insulin usually produces an increase in blood flow in human muscle (Allwood, Ginsburg & Paton, 1957). In the present experiments this result was not so clearly established, only three out of eleven experiments showing clear-cut increments. One reason for this may be found in the nature of the technique. Internal calorimetry is not a highly sensitive method. It is hard to detect changes in very low blood flows. Conductivity increments recorded from rat muscle are usually of the order of $0{\cdot}1–0{\cdot}5 \times 10^{-4}$ c.g.s. units. Occasionally, however, the recorder is in a situation which yields higher values—possibly this may be due to the proximity of large vessels or to some other unknown cause. The three examples which yielded positive blood-flow responses were all cases with relatively high resting conductivity increments. It is felt, therefore, that the present results are consistent with the view that in the rat, as in man, insulin may produce vasodilation in muscle. Combining the insulin with glucose uniformly produced no detectable effect on blood flow. Glucose alone had no effect that could be detected in seven experiments. In three out of the other four experiments it caused a rise in flow. In the remaining experiment it produced a fall. There is a superficial similarity between these and the findings obtained in liver but clearly further investigation—probably by other techniques—will be necessary before the mechanisms involved can be elucidated.

Heat production in muscle. The heat-production responses were much more consistent than the blood-flow results. Thus in nine out of eleven experiments there was a clear-cut rise in excess temperature, no change in the other two.

It is seen from Table 7 that the resting levels of heat production in muscle were very much lower than those in liver. Insulin produced rises

of from 0·036 to 0·28 arbitrary units per unit mass. In terms of percentage change this is a considerable increase. In absolute terms, however, compared with the liver values, they still remain slight.

The effect of glucose alone was to depress the resting metabolism still further in nine out of eleven experiments.

The effect of combining insulin with glucose, however, was to produce increments in heat output in every case of a very high order. Indeed, the results obtained suggest that glucose might even potentiate the insulin response in muscle. This is in striking contrast to the findings in liver.

Presumably, following insulin administration there is both increased glycogen break-down within the muscle and increased glucose utilization by the cells. The simultaneous administration of glucose will help to maintain glycogen stores that might otherwise become depleted—and possibly, thereby, potentiate the insulin effect. Glucose could be regarded as acting within the cell—whither, from experience with tissue space measurement, it is clear it will diffuse rapidly. It could be pictured as supplying the needs of the cell directly without the necessity for breaking down muscle glycogen. The present work shows that glucose inhibits muscle heat production. It might well be that in muscle, probably, as in liver, glycogen break-down is also a potent source of heat.

SUMMARY

1. 'Internal calorimetry' has been used for the determination of metabolic heat production as well as blood flow.

2. The calculation of 'excess temperature', i.e. the contribution of metabolism to recorded temperature changes, is described, together with experiments to justify the assumptions involved.

3. The method has been applied to a preliminary study of the effects of insulin and glucose on blood flow and metabolism in liver and muscle.

4. Resting heat production in rat liver may be expressed in arbitrary units which enable quantitative comparisons to be made. Resting heat production in liver appears to be about 95 times that in resting muscle.

5. Insulin (I.V.) produces a 44% increase in liver heat production and a 670% increase in muscle heat production.

6. Glucose (I.V.) depresses both liver and muscle heat production.

7. Combinations of insulin and glucose (I.V.) produce variable but usually slight effects in liver. In contrast, combinations of insulin and glucose (I.V.) result in a greatly increased heat production in muscle.

8. The possible relation of these effects to glycogen break-down is discussed.

9. In the liver it is confirmed that insulin usually produces vasodilatation which, however, is prevented by the simultaneous administration of glucose. Glucose alone, however, usually also causes vasodilatation.

10. Subject to the sensitivity limitations of 'internal calorimetry' in the measurement of muscle blood-flow change it has also been confirmed that insulin may produce vasodilatation in muscle, preventable by the simultaneous administration of glucose, but that glucose alone may sometimes also produce vasodilatation.

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CLOTTING ACTIVITY OF MATERNAL AND FOETAL SHEEP BLOOD

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Most of the blood clotting factors are proteins and are produced by the liver. However, it is undecided where Factor VIII (antihaemophilic factor) is produced. (Nomenclature recommended by the International Committee for the Standardization of the Nomenclature of Blood Clotting Factors (*Brit. med. J.* 1959, i, 1292–1294) has been adopted in this paper.) Foetal liver lacks certain enzymes essential for protein synthesis, namely, those which hydroxylate phenylalanine and oxidize tyrosine (Kenney, Reem & Kretchmer, 1958). Further, γ-globulins and antibodies, which are produced by extra-hepatic tissues (Coons, Leduc & Connolly, 1955; Dancis, Braverman & Lind, 1957; Ortega & Mellors, 1957), are either absent, or present in only very low concentrations, in the foetal plasma of some species (Moore, Du Pan & Buxton, 1949). If, as evidence suggests, the foetal organism cannot form antibodies it may be possible to introduce into it cells which produce Factor VIII in the hope that they will survive and produce Factor VIII in the growing child. If grafting of Factor-VIII-producing cells were successful in new-born male infants of mothers who are α-haemophilia carriers it should then be possible to prevent them from becoming haemophiliacs.

It is therefore essential to know the activity of clotting factors of foetal blood. Such data from sheep are given here.

METHODS

Healthy ewes of known date of conception were used. They were of Merino breed and were mated with Merino rams. The gestation period is 144–160 days. Seventeen animals were available but not all tests were carried out on each animal. The pregnant ewes at varying stages of gestation were anaesthetized with intravenous Nembutal or Pentothal (pentobarbitone, thiopentone; Abbott Laboratories) and the foetuses exposed by Caesarian section. With siliconed syringes blood was withdrawn from one of the cord vessels and from the jugular vein of the ewe.

Plasma was obtained by mixing 9 volumes of blood with 1 volume of 0·1 M sodium oxalate and centrifuging at 2000 *g* at 6° C for 20 min. Plasma specimens were kept on ice until tested. The euglobin fraction of plasma was prepared as described by Fantl & Ward (1957).

Serum for the thromboplastin generation test was obtained by allowing blood to clot at 37° C for 1 hr. However, in order to obtain serum free of prothrombin it was necessary to clot 2·5 ml. blood in the presence of 0·1 g of glass-wool.

Brain was dehydrated with acetone according to the method of Quick (1940) and a suspension (3 or 6 %) of the dry powder in 0·145 M-NaCl was prepared by frequent grinding at 37° C during 2 hr in a glass tube with a pestle.

Clotting time was measured at 37° C on 1·5 ml. of blood in glass and in siliconed tubes.

One-stage prothrombin tests were performed, (*a*) with human and sheep brain extracts according to the method of Quick (1938); (*b*) with Russell's viper venom and phospholipid essentially according to the procedure of Hobson & Witts (1941). Prothrombin assays were carried out by the technique of Fantl (1954). Two procedures were used: (*a*) with human or sheep brain suspension; (*b*) the brain suspension was replaced by Russell's viper venom and phospholipid.

In preliminary experiments it was noted that the thrombin yield depended upon plasma concentration in the reaction mixture. The lowest plasma concentration gave the highest yield. It could be shown that catechol, which is used in the assay procedure to counteract antithrombin activity, did not affect the yield in the concentration employed. Further, the plasma euglobulin fraction, which is free from antithrombin, behaved similarly to plasma in the prothrombin assays.

Factor V was determined by the ability of the test plasma to shorten the clotting time of oxalated human plasma stored for 14 days at room temperature, the pH being adjusted to 7·4 with carbon dioxide before use. The test was similar to that of Quick & Stefanini (1948).

The combined activity of Factor VII and Prower-Stuart factor in serum was measured according to the procedure of de Vries, Alexander & Goldstein (1949).

Thromboplastin generation tests were performed at 28° C essentially as described by Biggs & Douglas (1953), except that oxalated plasma treated with $BaSO_4$ at 6° C for 30 min (referred to hereafter as Ba-plasma) was used and a phospholipid suspension in veronal buffer, pH 7·4, made according to the method of Fantl (1958) and containing 140 μg phospholipids, was substituted for platelets.

Partial thromboplastin times were determined by the method of Langdell, Wagner & Brinkhous (1953) modified as follows: a mixture of 0·2 ml. oxalated plasma, 0·1 ml. phospholipid suspension in veronal buffer, pH 7·4, and 0·05 ml. 0·145 M-NaCl or the material to be tested were incubated at 37° C for 1 min; 0·2 ml. 0·025 M-$CaCl_2$ was then blown into the mixture and the clotting time observed.

Assay of Factor VIII was carried out with oxalated plasma from a patient with a congenital complete deficiency of Factor VIII, to which dilutions of sheep or human Ba-plasma were added in either the thromboplastin generation test or the partial thromboplastin time test; similar clotting times were taken to indicate similar factor VIII activities. With both tests it is possible to determine Factor VIII activity which differs by more than 50 % from that of normal human plasma, the concentrations of other factors being equal.

Factor IX activity was measured with the thromboplastin generation test of Biggs & Douglas (1953), with sheep Ba-plasma and serum in the following way: clotting times obtained with 0·06 ml. pregnant ewe serum were taken to indicate 100 % Factor IX activity. A series of dilutions of ewe serum in 0·145 M-NaCl was prepared and that dilution which gave the same clotting time as 0·06 ml. foetal serum was taken as a measure of foetal concentration of Factor IX in terms of maternal concentration. Such a technique will only give accurate results if (*a*) the dilution of the test serum is not very high, and (*b*) it is assumed that other serum factors apart from Factor IX required for thromboplastin formation are present in adequate concentration. It was necessary to adopt the above technique because Factor-IX-deficient human plasma or serum in combination with foetal sheep serum gave a lower Factor IX concentration for the foetal sheep serum

than did homologous mixtures. The thromboplastin generation test is not very sensitive to changes in Factor IX activity, deviations of less than approximately 80% from normal not being detectable.

Human, sheep and bovine thrombin preparations were made as described by Fantl (1958).

Dialysis was carried out at 3° C in cellulose tubing suspended in a solution of 0·02 M trisodium citrate, 0·025 M sodium chloride and $1{\cdot}33 \times 10^{-4}$ M citric acid, pH 7·3, I approximately 0·145. The thrombin preparations were dialysed for 44 hr, at least one change of solution being made during this period, and the plasmata for 21 hr, with at least two changes of solution.

Nitrogen (N) was determined by a micro-Kjeldahl procedure.

Fibrinogen was determined as fibrin in 1 ml. oxalated plasma by addition of 1 ml. 0·025 M-$CaCl_2$ and 1 ml. of a thrombin preparation. The N content of the clot was determined after washing in 0·145 M-NaCl and was converted to fibrin by a factor of 5·92.

Plasma protein was estimated by determination of N in trichloroacetic-acid-insoluble material and conversion to protein by a factor of 6·25.

Moving-boundary electrophoresis was carried out in a Hilger Tiselius apparatus, in veronal-citrate buffer, pH 8·5, $I = 0{\cdot}05$, at 5° C, or in a Perkin–Elmer Model 38A Tiselius apparatus with an interferometric optical system, in veronal buffer, pH 8·6, $I = 0{\cdot}1$, at 4° C.

Platelets were counted by a technique similar to that of Feissly & Lüdin (1949).

RESULTS

The thromboplastin components in the blood of pregnant ewes and their foetuses

Results of the estimation of Factor VIII activity in the plasma of ewes and their foetuses are given in Table 1.

TABLE 1. Factor VIII activity in plasma of pregnant ewes and their foetuses expressed in terms of an arbitrary activity of 100 for human plasma

Gestation time (days)	86	99	128	128	134	142	145
Pregnant ewe plasma (a)	2500	1000	350	500	900	1200	650
Foetal plasma (b)	200	100	200	400	200	500	750
$b/a \times 100$	8	10	57	80	22	42	115

It is apparent from these results that a very low Factor VIII activity was present in foetal plasma at a gestation time of 86 days, and it increased during pregnancy. The activities given in the table are expressed in terms of normal human plasma which is arbitrarily taken to have an activity of 100. The experiments were carried out during several seasons and the human plasma used as a comparison was not taken from the same person in each case. As the Factor VIII activity of normal human plasma varies between 80 and 200 this variation is reflected in the potencies of the sheep plasmata. Plasma of normal non-pregnant sheep varied in Factor VIII activity from 450 to 1000. The values found for pregnant ewes showed similar variation. It will be noted that the lowest Factor VIII activity of foetal sheep plasma was of the same order as that of adult human plasma. Additional information on the Factor VIII activity of foetal plasma is supplied by thromboplastin generation tests in which mixtures of either pregnant ewe Ba-plasma or foetal Ba-plasma with ewe serum or

foetal serum were used. Results are given in Table 2, and show that, with the same serum, Ba-plasma of ewe or foetus gave similar clotting times. This indicates that the Factor VIII activity of foetal sheep plasma is not significantly lower than that of the maternal plasma.

The results given in Table 1, obtained from experiments carried out with heterologous plasma mixtures, showed lower Factor VIII activity for foetal sheep plasma than those in Table 2. Apparently Factor VIII of foetal sheep plasma has less affinity to the other components of the thromboplastin complex of human plasma than to those of sheep plasma.

TABLE 2. Thromboplastin generation test of Ba-plasma of pregnant ewe and foetus and homologous serum

Gestation time (days)	Serum	Ba-plasma	Minimum clotting time (sec)	Incubation time (min) for minimum clotting time
86	Pregnant ewe	Pregnant ewe	13	4
		Foetus	12	< 3
	Foetus	Pregnant ewe	15	< 2·5
		Foetus	16	< 3
142	Pregnant ewe	Pregnant ewe	12	< 3
		Foetus	12	< 3
	Foetus	Pregnant ewe	18	5
		Foetus	18	4·5

TABLE 3. Factor IX activity in foetal sheep serum expressed as percentage of maternal serum activity in thromboplastin generation test

Gestation time (days)	65	72	77	86	92	99	128	128	145
Foetal Factor IX activity	5	15	15	25	25	30	20	10	30

Results of the estimation of Factor IX activity in the serum of foetuses are given in Table 3.

This table shows that at all periods of gestation the foetal serum has a considerably lower activity than that of the mother.

The prothrombin complex in sheep blood

The prothrombin activity of plasma, which includes the activity of accessory factors for thrombin formation, was determined in one-stage tests in the presence of homologous and heterologous brain extracts and also with Russell's viper venom and phospholipid. Results are summarized in Table 4. This shows that a brain extract gave shortest clotting times with homologous plasma. Human plasma was less reactive with sheep brain than was sheep plasma with human brain. Foetal sheep plasma gave longer clotting times than maternal plasma with both sheep and human brain extracts. Russell's viper venom did not differentiate sig-

nificantly between the species although somewhat longer clotting times with foetal plasma were observed.

In Table 5 are shown one-stage prothrombin times and prothrombin assays related to the age of the foetus. From these results it is seen that the one-stage prothrombin time of foetal plasma was at all gestation times longer than that of the maternal plasma. Prothrombin assays indicated a very low prothrombin concentration in foetal plasma at an age of 70 days rising to 60 % of the maternal plasma concentration towards the

TABLE 4. One-stage prothrombin times (sec)

Oxalated plasma of	Human brain	Sheep brain	Russell's viper venom + phospholipid
Man	16 (15–17)	27 (22–32)	7·5 (7–8·5)
Pregnant ewe	18 (17–19)	10·5 (8·5–13)	8·5 (7–10)
Foetal sheep	23 (19–26)	13·5 (11·5–18)	10 (7·5–11·5)

Means (ranges in parentheses).

TABLE 5. One-stage prothrombin times and prothrombin concentrations in plasma of pregnant ewes and their foetuses

Gestation time (days) ...		70	77	92	98	98	98	128	128	134	145
One-stage prothrombin time (sec)*	Pregnant ewe	10	8·5	10·5	10	10	11	9	10·5	13	12
	Foetus	17·5	14·5	12·5	12	13	11·5	11·5	15·5	14	13·5
Prothrombin† concentration in plasma (units/ml.)	Pregnant ewe (*a*)	400	360	260	340	460	250	430	540	370	430
	Foetus (*b*)	65	50	130	120	120	60	200	240	160	260
$b/a \times 100$		16	14	50	35	26	24	47	44	43	60

* Sheep brain extract and 0·01 M-$CaCl_2$ were used.

† The assays were carried out with sheep brain extract, and in some cases with 5 μg Russell's viper venom and 25 μg phospholipid in place of the brain extract

end of gestation. It was established that the values obtained for prothrombin concentration in foetal plasma represented the true prothrombin level, since addition of the accessory factors—Factor V in ewe Ba-plasma and Factor VII and Prower–Stuart factor in ewe serum—did not increase the thrombin yield. For comparison the prothrombin level found for non-pregnant sheep was 280–460 units/ml. plasma and for man 480–640 units/ml. plasma.

For conversion of prothrombin into thrombin adequate amounts of Factor V are essential. Factor V activities of plasma of pregnant ewes and their foetuses are given in Table 6. It is apparent from Table 6 that at all gestation times studied the Factor V activity of foetal plasma was approximately the same as that of the ewe. Normal human plasma showed considerably lower activity.

In addition to Factor V two more plasma factors are required for efficient thrombin formation from prothrombin; these are Factor VII and

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nificantly between the species although somewhat longer clotting times with foetal plasma were observed.

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Means (ranges in parentheses).

TABLE 5. One-stage prothrombin times and prothrombin concentrations in plasma of pregnant ewes and their foetuses

Gestation time (days) ...		70	77	92	98	98	98	128	128	134	145
One-stage prothrombin time (sec)*	Pregnant ewe	10	8·5	10·5	10	10	11	9	10·5	13	12
	Foetus	17·5	14·5	12·5	12	13	11·5	11·5	15·5	14	13·5
Prothrombin† concentration in plasma (units/ml.)	Pregnant ewe (*a*)	400	360	260	340	460	250	430	540	370	430
	Foetus (*b*)	65	50	130	120	120	60	200	240	160	260
$b/a \times 100$		16	14	50	35	26	24	47	44	43	60

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For conversion of prothrombin into thrombin adequate amounts of Factor V are essential. Factor V activities of plasma of pregnant ewes and their foetuses are given in Table 6. It is apparent from Table 6 that at all gestation times studied the Factor V activity of foetal plasma was approximately the same as that of the ewe. Normal human plasma showed considerably lower activity.

In addition to Factor V two more plasma factors are required for efficient thrombin formation from prothrombin; these are Factor VII and

the Prower–Stuart factor. They were determined together by the ability to shorten the clotting time of diluted sheep plasma in the presence of sheep brain extract. Results are given in Table 7 from which it can be seen that Factor VII activity of foetal serum runs roughly parallel to prothrombin concentration.

TABLE 6. Factor V activity in plasma of pregnant ewes and their foetuses expressed in terms of an arbitrary activity of 100 for human plasma

Gestation time (days) ...	85	99	128	128	134	145
Pregnant ewe plasma ...	750	350	500	650	1000	500
Foetal plasma ...	750	250	350	750	700	1000

TABLE 7. Combined Factor VII and Prower–Stuart factor activity in serum of foetal sheep expressed as percentage of maternal serum activity. (Prothrombin concentrations in the respective plasmata, expressed as percentage of maternal plasma prothrombin concentration, are given for comparison)

Gestation time (days)	72	86	99	128	145
Factor VII + Prower-Stuart factor	20	50	50	30	50
Prothrombin	—	40	36	47	60

Fibrinogen concentration and activity of plasma of ewe and foetus

Fibrinogen and total protein concentrations of plasma are recorded in Table 8, from which it can be seen that at a foetal age of 70 days the fibrinogen concentration in foetal plasma was 16 % of that of the maternal plasma and near term had reached 83 % of the maternal level. Over the same interval the total protein concentration of the foetal plasma increased from 34 to 90 % of that of the mother. These results are in agreement with the findings of Alexander, Nixon, Widdas & Wohlzogen (1958). Plasma of non-pregnant sheep contained 200–350 mg fibrinogen/100 ml.

The reactivity of the fibrinogen of human, pregnant ewe and foetal sheep plasmata was compared with different thrombin preparations under a variety of experimental conditions. The results are summarized in Table 9. These results were obtained with fresh oxalated plasmata and also with dialysed plasmata. In the latter case maternal and foetal specimens were used from three different ewes of gestation times 128, 128 and 134 days.

It can be seen that with human and pregnant ewe plasmata the various thrombin preparations showed only small differences in activity. However a calcium-free human thrombin preparation gave considerably lower activity with foetal sheep plasma than with human or pregnant ewe plasma.

The addition of calcium ions had a greater shortening influence on the thrombin clotting time of the foetal plasma than on the other two plasmata. Thus in the presence of optimal calcium-ion concentration human throm-

bin produced practically identical clotting times in human, ewe and foetal plasmata. Although addition of calcium ions also shortened the thrombin clotting times when sheep or bovine thrombin was used the effect was not so pronounced as in the case of human thrombin.

The thrombin clotting time is dependent on fibrinogen concentration, but over the range of fibrinogen concentrations from 150 to 400 mg/100 ml. it does not vary significantly. Since all the plasmata used in the experiments shown in Table 9 had fibrinogen concentrations within this range it is unlikely that any differences in thrombin clotting time are due to markedly different fibrinogen levels.

TABLE 8. Fibrinogen and protein concentration in plasma of pregnant ewes and their foetuses

Gestation time (days) ...		70	77	86	98–99 (4)*	128 (2)*	134	142	145
Fibrinogen concentration in plasma (mg/100 ml.)	Ewe (*a*)	310	330	530	315–410	235–425	430	440	310
	Foetus (*b*)	50	70	155	140–210	195–255	145	225	230
$b/a \times 100$		16	21	29	34–67	60–83	34	51	74
Total protein concentration in plasma (g/100 ml.)	Ewe (*c*)	5·8	6·2	7·6	7·2–8·2	6·5–7·5	8·3	7·7	5·9
	Foetus (*d*)	2·0	2·4	2·7	2·8–3·2	4·5–4·6	3·8	4·8	5·3
$d/c \times 100$		34	39	36	35–42	60–71	46	62	90

* Where determinations were made on more than one ewe and foetus of a given gestation period the number of pregnant ewes is stated in parentheses.

TABLE 9. Thrombin activity with plasmata of man, pregnant ewe and foetal sheep

Plasma	Addition	Thrombin: Human	Thrombin: Bovine	Thrombin: Sheep
Human	NaCl	1	1	1
	$CaCl_2$	2·7	3·4	2·0
Pregnant ewe	NaCl	1	1·7	1·1
	$CaCl_2$	2·7	3·6	2·2
Foetal sheep	NaCl	0·2	1·5	1·1
	$CaCl_2$	2·7	4·0	2·3

0·2 ml. of either 0·075 M-NaCl or 0·025 M-$CaCl_2$ was added to 0·1 ml. plasma and 0·1 ml. veronal buffer, pH 7·3. 0·1 ml. thrombin preparation was then added at 36° C. Clotting times were converted into thrombin units. The results were recalculated taking the result obtained with human plasma with addition of NaCl as 1. Comparisons can only be made within each column.

The observations indicate that fibrinogen of foetal sheep plasma has properties different from that of maternal plasma. This is also seen in the behaviour of the blood clot. Whereas maternal sheep blood showed good clot retraction and little erythrocyte fall-out from the clot in a subsequent incubation period, foetal blood clots showed little or no retraction and a high fall-out of erythrocytes. The poor clot retraction could be due to a deficiency in the foetal platelets which, however, were present in

adequate number, as can be seen from Table 10. The erythrocyte fall-out is probably due to a structural weakness of the foetal fibrin which is unable to retain the erythrocyte mass. Fibrinolysis, which could be a cause of this high fall-out from the clot, was not detected in the foetal blood.

Whole blood clotting time

Determinations of whole blood clotting time are presented in Table 11 and show very little difference between foetal and maternal blood. The somewhat shorter clotting times of foetal blood are probably due to contamination of foetal blood with tisue thromboplastin during collection. The fact that clotting times in silicone were longer than those in glass for both maternal and foetal blood suggests that in both cases adequate concentrations of the contact factor (Hageman factor) are present.

TABLE 10. Platelet counts in blood of pregnant ewes and their foetuses

Gestation time (days) ...		65	72	77	86	92	98	98	128	128	134	145
Platelets $\times 10^{-3}$ per mm^3 blood	Ewe	420	430	480	640	610	480	450	550	790	430	630
	Foetus	200	240	470	370	560	950	670	780	700	580	820

TABLE 11. Clotting time (min) in glass and in silicone of blood from pregnant ewes and their foetuses

Gestation time (days) ...		70	92	98*	99	128	134	145
Pregnant ewe	Glass	7	7	7–13	21	12	15	—
	Silicone	—	—	—	38	73	35	—
Foetus	Glass	7	6	6–7	—	15	14	13
	Silicone	—	—	—	—	50	26	55

* Determinations were made on the blood from three pregnant ewes and foetuses of 98 days gestation.

Electrophoresis of plasma and serum

γ-Globulins are produced by the reticulo-endothelial system and it is possible that this is also the site for Factor VIII synthesis. Therefore electrophoresis of maternal and foetal sheep plasmata was carried out. A copy of a photographic schlieren pattern of both plasma and serum is shown in Fig. 1. The electrophoretic patterns indicate that the foetal plasma and serum were free from γ-globulin (component γ). Minor variations from the patterns in Fig. 1 were found on electrophoresis of other pregnant ewe and foetal sheep plasmata and sera, and from examination of some foetal serum patterns obtained by use of an interferometric optical system it appeared that a trace of γ-globulin may have been present.

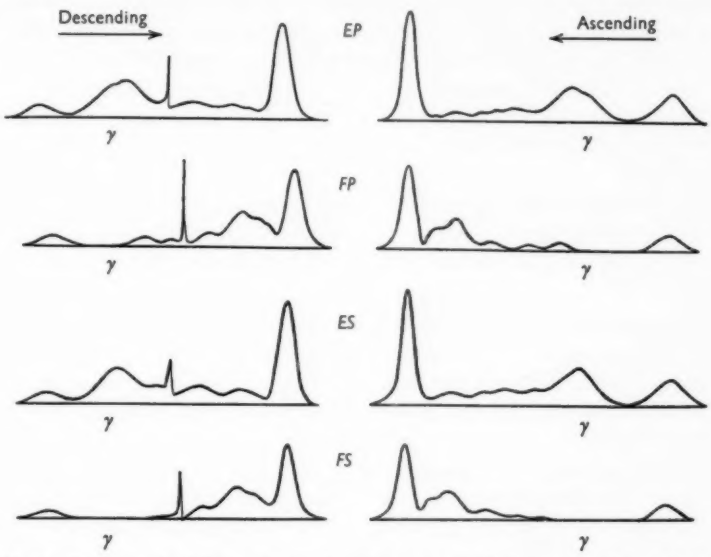

Fig. 1. Tracings of schlieren electrophoretic patterns of plasmata and sera from pregnant ewe and foetus of 98 days gestation. *EP* ewe plasma, *FP* foetal plasma, *ES* ewe serum, *FS* foetal serum. Conditions: veronal-citrate buffer, pH 8·5, $I = 0{\cdot}05$, 5° C. The patterns on the right are from the ascending boundaries, those on the left from the descending boundaries.

DISCUSSION

Regarding the presence of clotting factors in foetal blood, the possibility of production within the foetal organism and circulation or of transfer from the maternal circulation has to be considered. It cannot be stated whether Factor VIII is produced by the foetus or placenta, or is transferred from the ewe to the foetus. Some evidence for the production of Factor VIII in the foetal organism is given by the fact that Factor VIII in foetal plasma has a higher affinity, relative to that of Factor VIII in the maternal plasma, for the other components of the blood thromboplastin complex when these are homologous than when they are of human origin. It is interesting to note that the Factor VIII activity of the plasma of the youngest foetus (86 days) was as high as that of plasma of adult man.

The present experiments do not indicate the source of Factor VIII in plasma. A reduction of Factor VIII in experimental animals has been reported to occur following administration of ethionine (Pool & Spaet, 1954) and liver damage by hepatotoxins (Penick, Roberts, Webster & Brinkhous, 1958). This could be due to interference with protein synthesis

and does not indicate a specific site for Factor VIII synthesis, which may occur in the reticulo–endothelial system (Pool & Spaet, 1954). γ-Globulins were absent from foetal sheep serum, or at most, present in trace amounts. Therefore the reticulo-endothelial system of the sheep foetus is not fully developed, but while it is not able to produce γ-globulins it may be able to perform some of the other functions of the adult reticulo-endothelial system.

Evidence that synthesis of Factor VIII is not associated with γ-globulin synthesis in man is provided by the finding of Frick & Good (1956) that agammaglobulinaemia is not accompanied by reduced levels of Factor VIII.

The comparatively high Factor V activity of foetal plasma is remarkable. The available evidence indicates that this factor is produced by the liver in adults (Sykes, Seegers & Ware, 1948) but the source of Factor V in foetal plasma is undecided. In view of the fact that Factor V activity of foetal sheep plasma is of the same order as that of the pregnant ewe it is possible that Factor V is transferred from the maternal circulation to the foetus. Quick, Murat, Hussey & Burgess (1952) and Fresh, Ferguson, Stamey, Morgan & Lewis (1957) found Factor V levels in cord blood from new-born infants to be equal to or greater than the normal adult human level. In both full term and premature new-borns van Creveld, Paulssen & Teng (1952) observed Factor V concentrations in the normal adult range.

The prothrombin concentration of foetal sheep plasma was very low at 70 days and rose to 60% of that of the maternal plasma towards the end of gestation.

In full-term new-born humans mean plasma prothrombin concentrations varying from approximately 25 to 45% of the normal adult mean have been reported (Brinkhous, Smith & Warner, 1937; Loeliger & Koller, 1952; van Creveld, Paulssen & Teng, 1952; Fresh *et al.* 1957).

In foetal sheep serum the combined activity of Factor VII and Prower-Stuart factor, which were not determined separately in the present study, was found to be similar to the concentration of prothrombin and there was some parallelism between the levels of these factors and foetal age. Larrieu, Soulier & Minkowski (1952), van Creveld, Paulssen, Ens, van der Meij, Versteeg & Versteegh (1954), and Fresh *et al.* (1957) found the mean Factor VII content of cord blood from full-term infants to be approximately 40% of the mean normal adult level.

The fact that the activity of prothrombin, Factor VII and Prower-Stuart factor in foetal sheep blood is lower than in the maternal blood and the approximate correlation between the foetal blood levels and age may reflect the diminished ability of the foetal liver to produce these clotting factors.

Prothrombin and Factors VII and IX have a number of properties in common and may be chemically related. The much lower activity of Factor IX in foetal serum is therefore surprising. Perhaps the enzyme system required for synthesis of Factor IX is more complex than those involved in prothrombin and Factor VII synthesis and is not well developed in the foetal liver. Barkhan (1957) reported that sera from cord blood of new-born infants had a Factor IX activity of 19–91% (mean 49%) compared with a mean activity of 108% for normal human adult serum.

The fibrinogen concentration in foetal sheep plasma was 50 mg/100 ml. in the youngest foetus on which a determination was carried out (70 days); near term it had reached 230 mg/100 ml. which is approximately 80% of the concentration in ewe plasma.

In new-born lambs Field, Spero & Link (1951) found an average of 226 mg fibrinogen and 6·15 g total protein/100 ml. plasma.

In eight human foetuses, from 15 to 20 weeks of age, Vahlquist, Westberg & de las Heras (1953) found 50–290 (mean 180) mg fibrinogen/100 ml. plasma, and 140–700 (mean 390) mg/100 ml. plasma in the cord blood of fourteen full-term infants. Taylor (1957) has summarized a number of studies on fibrinogen concentration in the normal new-born infant, and has reported his own findings of 192–398 (mean 231) mg/100 ml. plasma compared with 305–674 (mean 440) mg/100 ml. in maternal plasma and 217–354 (mean 280) mg/100 ml. for normal adults.

Foetal sheep fibrinogen showed the same behaviour as maternal fibrinogen towards sheep and bovine thrombin but gave longer clotting times with thrombin of human origin. However, on addition of calcium ions, which shortened the clotting time in all cases, this different behaviour of human thrombin with foetal sheep fibrinogen was abolished.

A prolonged thrombin clotting time has been noticed with fibrinogen of new-born infants (Biggs, 1951; Larrieu *et al.* 1952; Ferguson, Fresh & Lewis, 1955; Vecchio & Schettini, 1957), and Jayle, Badin & Ottolenghi-Preti (1949) and Burstein, Lewi & Walter (1954) have found that certain physical properties of the fibrin clot from the plasma of new-born infants are different from those of normal adult fibrin. The finding of a difference in activity of the foetal and maternal fibrinogen indicates that fibrinogen in the foetus is produced in the foetal circulation rather than being transferred from the ewe.

Although the placenta of the sheep is of the syndesmochorial type and thus differs structurally from the human placenta, which is of the more permeable haemochorial type, it appears from a comparison of the presented results with the findings in human blood that the respective activities of the clotting factors in the blood of mother, foetus and normal

adult are similar in the two species. Certain speculations about blood coagulation in the human infant would therefore seem justified.

Determination of Factors VII, IX, and prothrombin in the blood of a new-born infant does not permit one to predict whether a haemorrhagic tendency due to a deficiency of one or more of these factors will develop later in life as the activity of these factors is low in the normal new-born and increases with age. On the other hand the activity of Factor VIII in the blood of the normal new-born is as high as in the normal adult (Fresh, Ferguson & Lewis, 1956; van Creveld, Nagel, Nijenhuis, Miranda & Tjon Sien Kie, 1954).

We found 50–75 % of the normal adult factor VIII activity in the cord blood of a new-born baby in whose pedigree there was no record of Factor VIII deficiency. As far as we are aware no observations of Factor VIII levels in new-born infants of carriers of Factor VIII deficiency has yet been made, but it would seem that a low Factor VIII activity in the blood of a new-born infant would indicate a permanent Factor VIII deficiency and measures to induce Factor VIII production as suggested in the introduction would be justified.

SUMMARY

1. Factor VIII activity is detectable in foetal sheep plasma at an early stage of gestation and rises to adult levels towards the end of gestation. At all gestation times Factor VIII activity of foetal sheep plasma is at least as high as that of adult human plasma.

2. Factor V activity of foetal sheep plasma is at all gestation times as high as that of maternal plasma. Factor V activity of sheep plasma is higher than that of man.

3. Prothrombin activity of foetal sheep plasma is low at early stages of gestation and rises to approximately 60 % of that of maternal plasma near term. Normal sheep plasma contains approximately 70 % of the prothrombin of normal human plasma.

4. In the serum of foetal sheep, Factor VII and Prower–Stuart factor, measured together, show some parallelism with the prothrombin concentration of plasma.

5. Factor IX activity of foetal sheep serum is at all foetal ages low compared with that of the maternal serum.

6. Fibrinogen concentration of foetal sheep plasma is low at early stages of gestation and rises to approximately 80 % of that of the maternal plasma near term.

7. Foetal sheep fibrinogen shows different activity from that of the maternal fibrinogen.

We wish to thank Dr R. H. Watson, of the Commonwealth Scientific and Industrial Research Organization, who made available the pregnant ewes of known date of conception and supplied information on the gestation period; Mr H. M. Radford, of the same department, who performed the surgical procedures necessary to obtain blood samples in the earlier experiments; Mr I. A. L. Ferguson and Mr G. R. Stirling, of the Clinical Research Unit, Alfred Hospital, Melbourne, who carried out the necessary surgical procedures in later experiments; Mr F. J. Dempster, of the Commonwealth Serum Laboratories, Parkville, Victoria, and Dr C. C. Curtain, of the Baker Medical Research Institute, Alfred Hospital, Melbourne, who performed moving boundary electrophoresis on plasma and serum samples. Part of the expenses of this investigation were defrayed by a grant from the National Health and Medical Research Council, Australia.

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TRANSAMINASE ACTIVITY IN THE PERFUSED RAT HEART

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Use of the isolated heart as a preparation for the study of amino-acid metabolism has so far been restricted to determination of ammonia and carbon dioxide production from amino acids (Clark, Gaddie & Stewart, 1931; Cruickshank & McClure, 1936; Clark, Eggleton, Eggleton, Gaddie & Stewart, 1938; Lorber & Olsen, 1946; Clarke, 1957). It seemed possible that an investigation of transamination in such a preparation might give further information, especially as work with homogenates of heart muscle has shown that enzymes catalysing more than 20 transaminase reactions are present in pig and rat heart muscle (Cammarata & Cohen, 1950). The results of the experiments reported in this communication have shown that interconversion of glutamic and aspartic acids, but of no other amino acids, can be demonstrated by analysis of the perfusate of the isolated rat heart.

METHODS

Animals. Female albino rats were used, 4–6 months old, weight 200 ± 20 g, bred in the department and fed on commercial rat cake (Diet 41, Parkes, 1946).

Perfusion fluid. Krebs's bicarbonate solution was used, prepared according to the method of Umbreit, Burris & Stauffer (1949), omitting potassium phosphate and with additions as described in the text. (All amino and keto acids were neutralized with NaOH.) This medium was always equilibrated in the perfusion apparatus with a gas mixture of 5 % CO_2 in oxygen.

Perfusion method. The apparatus used was similar to that described by Bleehen & Fisher (1954). Instead of surrounding the whole apparatus with a temperature-controlled water jacket, only the perfusate about to enter the heart and that in the heart chamber was maintained at 37° C by surrounding the down-flow tube and heart chamber with a water jacket. The heart itself was kept warm and moist by immersion in the perfusate in the heart chamber. As the gas injector, return tube and perfusate reservoir were at room temperature, there was little evaporation, and this was further reduced by covering the surfaces of the perfusate reservoir and heart chamber with pads of damp filter paper. The decrease in perfusate volume, estimated by determination of the chloride content, was found to be 0·15 ml./hr. A narrow glass rod introduced into the perfusate chamber outside the soxhlet filter facilitated the release of gas bubbles which occasionally became trapped beneath the filter at the mouth of the down-flow tube. No difficulty was encountered with frothing and the anti-frothing device described by Bleehen & Fisher (1954) was omitted.

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Experimental procedure

60 ml. of medium was circulated through the perfusion apparatus for at least 15 min to allow adequate filtration and gas equilibration before including the heart in the circuit. The animal was killed by breaking the neck and the heart was rapidly removed to a dish of cold medium, thus reducing the oxygen consumption of the tissue and rendering it quiescent for ease in cannulation. The cannula, filled with filtered medium withdrawn from the perfusion apparatus, was tied into the aorta (a procedure easily performed single handed if the cannula is temporarily secured to the working surface with a piece of 'Sellotape'), and the cannula with heart attached was secured to the down-flow tube of the perfusion apparatus. Before placing the heart in the heart chamber, and so closing the circuit, it was washed free from blood, the washings collected and the volume measured. The volume of the perfusate was readjusted to 60 ml. with fresh medium added to the perfusate reservoir, so that it passed through the soxhlet filter before reaching the heart. In most experiments the heart was perfused for 2 hr and samples of perfusate withdrawn from the perfusate reservoir were analysed by the methods described below. In some experiments the amino-acid content of the heart as well as that of the perfusate was determined. Hearts remained actively beating at a rate of 250–300 beats/min for up to 4 hr. Glucose utilization, calculated from that used during perfusion for 2 hr, was 72 ± 29 (S.D.) μmoles/g dry wt./hr when the heart was perfused with 60 ml. medium containing 1 m-mole glucose.

Analysis of the heart

Estimation of amino acids. At the end of an experiment hearts were re-perfused with fresh Krebs's solution (without added substrates) for 2 min, blotted dry and then frozen in solid CO_2 and fragmented by crushing in a press. The frozen powdered tissue was ground with 4·5 ml. ice-cold water and the supernatant, collected after centrifugation, heated in a boiling water-bath for 5 min to coagulate the soluble protein. The addition of one drop N-acetic acid to the supernatant while heating reduced the pH to about 5·4 and assisted coagulation. After further centrifugation, amino acids were separated and identified on a two-dimensional chromatogram, with 80% (w/v) phenol in water and *n*-butanol:acetic acid:water (4:1:5) as solvents.

For quantitative analysis a method was used adapted from that described by Bronk & Fisher (1956). 20 μl. spots of the heart extract were applied to Whatman No. 1 paper and the amino acids separated, with 80% (w/v) phenol in water as the solvent. After drying, the paper was dipped in a solution of 0·4% (w/v) ninhydrin in *n*-butanol and allowed to dry. The colour was developed by heating at 60° C for 30 min in an oven. The paper was then sprayed with a solution made by diluting 2 ml. saturated aqueous $Cu(NO_3)_2$ and 0·4 ml. 10% (v/v) HNO_3 to 100 ml. with *n*-butanol, and allowed to dry. The coloured spots were cut out, the colour eluted with 3 ml. methanol, and the extinctions of the methanol solutions measured at 510 mμ. Linear results were obtained with aqueous solutions of each amino acid over a concentration range of 0–500 μmoles/100 ml. 10 μl. samples of standard amino-acid solutions were applied to the chromatograph paper and taken through the whole analysis as described above. The colour intensity of the amino-acid spots varied slightly with different ninhydrin solutions, which were prepared fresh each week, and standards of each amino acid were included in each set of heart estimations. By this method it was possible to estimate the amount of individual amino acids in the heart to the nearest 25 μmoles/100 ml. heart water.

Estimation of the volume of extracellular water. The mean chloride content of a number of hearts, after perfusion with Kreb's solution containing 4·55 mg Cl^-/ml., was 1·65 mg, corresponding to a chloride space of 0·36 ml. R. B. Fisher & P. Zachariah (personal communication) have found a similar value (0·359 ml.) for the chloride space of hearts perfused for 15–120 min; but these authors consider that the raffinose space, which they find

to be 0·281 ml., provides a better estimate of the true extracellular space. The volume of the extracellular space of the hearts used in these experiments has therefore been taken to be 0·28 ml.

Analysis of the perfusate

Chromatographic separation of amino acids. Amino acids in the perfusate were separated and identified by one-dimensional chromatography, with 80 % (w/v) phenol in water as the solvent, and either strips of Whatman No. 4 or 54 paper, or discs of Whatman No. 1 paper (Proom & Woiwood, 1951). After drying, the paper was sprayed with 0·1 % (w/v) ninhydrin in *n*-butanol, and the amino-acid spots were developed by heating at 100° C for 15 min.

Oxaloacetic acid was estimated by the method of Greenwood & Greenbaum (1953), *glucose* by that of Hagedorn & Jenson (1923), *ammonia* and *urea* by those of Conway & O'Malley (1942).

Glutamic acid was estimated by decarboxylation with *Clostridium welchii* (Gale, 1945). Bacterial suspensions were prepared from stock culture of *C. welchii*, strain S.R.12, N.C.T.C. 6784, maintained on Robertson's cooked-meat medium. The *C. welchii* was grown for 16 hr at 37° C in 2 l. Lemco broth containing 2 % (w/v) glucose, and harvested by centrifuging. The cells were washed once in distilled water and resuspended in 50 ml. 0·1 M acetate buffer, pH 4·4. To estimate glutamic acid, a 2 ml. sample of perfusate was added to 1 ml. 0·1 M acetate buffer in the main compartment of a Warburg flask and the pH brought back to 4·4 by the addition of 2–3 drops of 2 N acetic acid. 0·5 ml. of the bacterial suspension was placed in the side-arm of the flask and, after equilibration at 37° C, tipped into the main compartment. Incubation was continued until no more CO_2 was evolved or for 1½ hr, whichever was the shorter period. Decarboxylation of glutamic acid by this method is rapid and complete in about 30 min. A mixture of aspartic and oxaloacetic or α-oxoglutaric acids in the presence of *C. welchii* also produces a slow evolution of CO_2 with the concurrent formation of alanine (Meister, Sober & Tice, 1951; Hicks, 1955). In the conditions of these experiments the evolution of CO_2 continued at a slow steady rate for more than 2 hr, so that it was possible to estimate glutamic acid in the presence of aspartic acid and a keto acid by recording the CO_2 formation well beyond the time at which all the glutamic acid was decarboxylated. When the curve formed by plotting the CO_2 evolution against time was extrapolated backwards, it cut the ordinate at the value equal to the CO_2 production due to the glutamic acid content of the sample. Experiment showed this method to have an accuracy of ±5 μmoles/60 ml. perfusate.

Extractable transaminase activity. The perfusate was incubated at 37° C with 200 μmoles each of aspartic and α-oxoglutaric acid and the rate of glutamic formation estimated by quantitative decarboxylation.

RESULTS

Detection of transaminase activity

Hearts were perfused for 2 hr with 60 ml. medium containing approximately 200 μmoles each of amino and keto acid. This amino-acid level was chosen to give an amino N concentration in the perfusate of approximately 5 mg/100 ml., within the normal range for mammalian blood (4–6 mg/100 ml.). Reversible transamination was found to occur between aspartic and glutamic acids but not between other pairs of amino and keto acids (Table 1).

This lack of other transaminase activity might have been caused by inability of the acids to penetrate the heart cells. The amino-acid content of a number of hearts was therefore examined before and after perfusion

with amino acids. The initial level was determined on hearts which had been perfused for 2 min with amino-acid-free Ringer's solution to remove blood from the heart chambers and the coronary circulation. Considerable amounts of taurine, glutamic acid, glutamine, alanine and aspartic acid were present (Fig. 1*a*), as reported by Roberts, Lowe, Chanin & Jelinek (1957). After perfusion for 1 hr with an amino-acid-free medium, aspartic acid was no longer present and the glutamine and alanine content was reduced (Fig. 1*b*). If alanine were included in the perfusate, the aspartic

TABLE 1. Amino acids detected in the perfusate after perfusion of the isolated rat heart for 2 hr with 60 ml. Krebs's bicarbonate solution containing 1 m-mole glucose and 200 μmoles each of amino and keto acids as shown

Acids added to the perfusate		New amino acid detected in the perfusate
L-aspartic	α-oxoglutaric	glutamic
L-aspartic	pyruvic	none
L-glutamic	oxaloacetic	aspartic
L-glutamic	pyruvic	none
L-alanine	oxaloacetic	none
L-alanine	α-oxoglutaric	none
glycine	α-oxoglutaric	none
DL-valine	α-oxoglutaric	none
L-leucine	α-oxoglutaric	none
L-phenyl alanine	α-oxoglutaric	none
L-histidine	α-oxoglutaric	none

acid and glutamine content of the hearts had decreased as before after 2 hr, but the alanine content had greatly increased (Fig. 1*c*). Leucine, when added to the perfusate, was present in the heart after perfusion (Fig. 1*d*).

An approximate estimate was made of the amino-acid content of the heart cells before and after perfusion. The extracellular amino-acid content was calculated for an extracellular space of 0·28 ml. on the assumption that the amino-acid concentration was the same as that in the perfusion fluid, and this value was subtracted from the total amino acid found in the heart. For hearts perfused with medium containing amino acids, intracellular amino-acid values calculated in this way are probably too low, since the hearts were re-perfused for 2 min with amino-acid-free medium and some amino acid may have diffused out from the extracellular space. In spite of this underestimate, the amounts of leucine and of aspartic acid found in the heart cells after perfusion for 2 hr with media containing these acids were higher than after perfusion with an amino-acid-free medium (Table 2). In these experiments the amounts of alanine and glutamine present in the hearts were not determined separately, since the acids were eluted from one-dimensional chromatograms on which these substances run to the same place. However, as the amount of glutamine present in

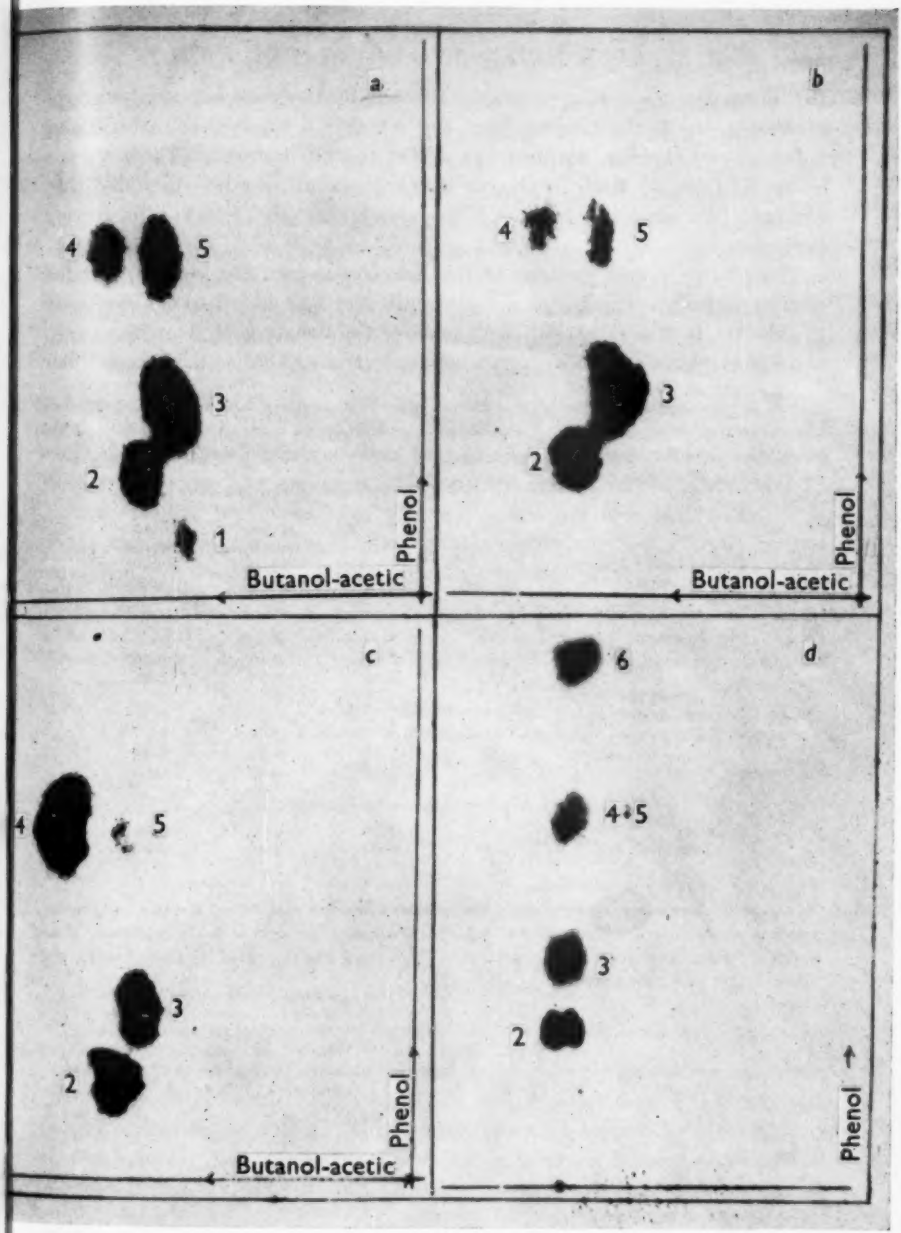

Fig. 1. Chromatograms prepared from extracts of isolated rat hearts perfused with 60 ml. Krebs's bicarbonate solution. *a–c*, two-dimensional, with butanol-acetic acid and phenol as solvents; *d*, one-dimensional, with phenol as solvent. *a*, heart perfused 2 min with amino-acid-free Ringer's solution; *b*, heart perfused 60 min with amino-acid-free Ringer's solution; *c*, heart perfused 60 min with Ringer's solution containing alanine; *d*, heart perfused 60 min with Ringer's solution containing leucine. 1 = aspartic acid; 2 = glutamic acid; 3 = taurine; 4 = alanine; 5 = glutamine; 6 = leucine.

the whole heart is not appreciably affected by the presence or absence of alanine in the perfusate (compare Fig. 1*b* and *c*) the higher intracellular value observed when alanine was added to the perfusate (Table 2) was essentially due to a rise in alanine content, and it may be concluded that alanine, like aspartic acid and leucine, enters the heart cells during perfusion.

The glutamic acid content of the hearts was initially high, and it fell during perfusion whether or not glutamic acid was added to the perfusate (Table 3). It was not possible to perfuse the hearts with a medium containing glutamic acid at as high a concentration as that of the intracelluiar

TABLE 2. Amino-acid content of rat hearts after perfusion for 2 hr with 60 ml. Krebs's bicarbonate solution containing 1 m-mole glucose and amino acids as shown. For method of calculating amino-acid content of extra- and intracellular water see text. (Mean values for triplicate experiments)

		Amino-acid content of hearts		
Amino acid added to perfusate	(μmoles)	Whole heart (μmoles)	Extra-cellular water (μmoles)	Intra-cellular water (μmoles)
	(*a*) Leucine content			
None		0	0	0
L-leucine	300	2·66	1·40	1·26
	375	3·06	1·75	1·31
	(*b*) Aspartic-acid content			
None		0	0	0
L-aspartic acid	150	1·13	0·70	0·43
	210	1·65	0·97	0·68
	(*c*) Alanine + glutamine content			
None		3·20	0	3·20
L-alanine	300	6·35	1·40	4·95
	315	7·20	1·47	5·73

TABLE 3. Glutamic-acid content of rat hearts after perfusion with 60 ml. Krebs's bicarbonate solution containing 1 m-mole glucose together with amino and keto acids as shown. Mean and S.E. for six experiments. For method of calculating glutamic acid content of extra- and intracellular water see text

			Glutamic-acid content of hearts		
Acids added to perfusate	(μmoles)	Perfusion time (min)	Whole heart (μmoles)	Extra-cellular water (μmoles)	Intra-cellular water (μmoles)
None		2	6·77 ± 0·21	0	6·77
None		60	5·54 ± 0·11	0	5·54
None		120	5·07 ± 0·07	0	5·07
L-glutamic	300	120	5·92 ± 0·09	1·40	4·50
	450	120	6·13 ± 0·14	2·10	4·02
L-aspartic + α-oxoglutaric	220 / 200	120	5·80 ± 0·06	0·31*	5·49

* Glutamic-acid content of perfusate, determined after perfusion for 2 hr, 67 μmoles in 60 ml.

water, since at concentrations above 7·5 mM (450 μmoles in 60 ml.) contractions were arrested. Although the glutamic acid content of the whole heart was increased by perfusing the heart with glutamic acid, the intracellular content apparently decreased. This anomalous result is likely to be due to over-estimation of the extracellular water content, as explained above. When, however, glutamic acid was formed during perfusion by transamination from aspartic to α-oxoglutaric acid, in spite of the low concentration of glutamic acid in the perfusate, the amount in the intracellular water was higher than in hearts perfused for the same time with an amino-acid-free medium or with one containing glutamic acid alone.

Glutamic/aspartic acid transamination

The amount of glutamic/aspartic transamination which occurred during perfusion was investigated by determination of the glutamic acid content of the perfusate. No glutamic acid was metabolized when it alone was added to the medium and this result was not affected by the omission or inclusion of glucose or lactate in the perfusate.

TABLE 4. Glutamic-acid content of the perfusate after perfusion of the isolated rat heart for 2 hr with 60 ml. Krebs's bicarbonate solution containing 1 m-mole glucose or 0·33 m-moles lactate and aspartic and α-oxoglutaric acids as shown. (Mean of duplicate experiments)

Acids added to the perfusate		Glutamic acid produced during perfusion
L-aspartic acid 225 (μmoles)	+ α-oxoglutaric acid (μmoles)	
	40	30
	90	65
	130	85
	160	80
	300	85
α-oxoglutaric acid 205 (μmoles)	+ L-aspartic acid (μmoles)	
	110	45
	225	70 ± 10*
	330	70
	480	80

* Mean value and S.E. for 24 experiments at these concentrations.

When oxaloacetic acid was added to the perfusate together with 205 μmoles of glutamic acid, 40 μmoles of the latter was transaminated during perfusion for 2 hr and this amount was not altered by variation of the oxaloacetic acid content of the medium between 90 and 630 μmoles. When the reaction was followed in the reverse direction the maximum amount of glutamic acid, 70 ± 10 μmoles, was formed when 225 μmoles aspartic acid and 90 μmoles α-oxoglutaric acids were added to the perfusate (Table 4). Increase in the concentration of either aspartic or α-oxoglutaric acid did not increase glutamic acid production. Addition to or

omission of glucose or of lactate from the perfusion did not affect the result.

The rate of formation of glutamic acid was followed by two methods. In the first, in a series of experiments, perfusion was stopped after different times and portions of the whole perfusate were analysed; in the second method samples of perfusate were removed and analysed at intervals during a single experiment. Although both methods are open to objection, the first because there were variations between individual hearts and the second because the perfusate was reduced in volume during an experiment,

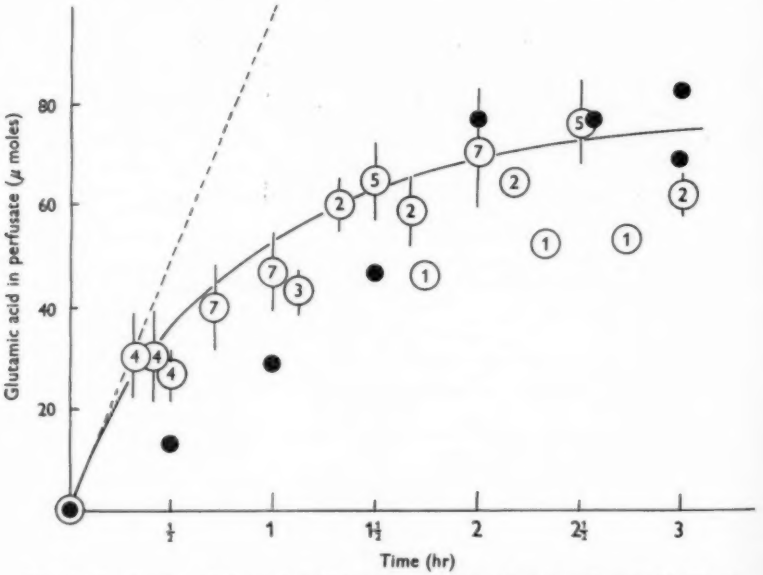

Fig. 2. Production of glutamic acid by the isolated rat heart perfused with 60 ml. Krebs's bicarbonate solution containing 225 μmoles aspartic acid, 205 μmoles α-oxoglutaric acid and either 1 m-mole glucose or 0·3 m-mole lactate. ●, Eight single experiments, each stopped at a different time; ◇, twelve experiments in which samples were taken from each heart at various intervals of time; the number of readings averaged for each point and the standard deviation are shown.

both showed that glutamic acid production ceased after 1½–2 hr (Fig. 2). The initial rate of glutamic acid production was 100 μmoles/hr (Fig. 2) which corresponds to a transaminase activity of $Q_T = 15$ for hearts with a mean dry weight of 150 mg. Measurement of the amount of oxaloacetic acid produced during perfusion showed that at all times the amounts of glutamic and of oxaloacetic acids in the perfusate were equivalent (Table 5). The cessation of glutamic acid production after 1½–2 hr when

only about 33 % of the aspartic acid had been converted to glutamic acid, suggested that an equilibrium mixture of amino and keto acid might have been reached; but when the reaction was followed in the opposite direction only 20 % of added glutamic acid was utilized, so that it is evident that the steady states attained did not reflect a true reaction equilibrium.

Cardiac damage is known to result in secretion of glutamic/aspartic transaminase into serum (Agress, Jacobs, Glassner, Lederer, Clark, Wroblewski, Karmen & LaDue, 1955) and it seemed possible that the activity measured during perfusion might be due to release of this enzyme into the perfusate. For this reason the transaminase activity of amino-acid-free media, circulated through the heart for times varying from 15 to

TABLE 5. Glutamic and oxaloacetic-acid content of the perfusate after perfusion of the isolated rat heart for various times with 60 ml. Krebs's bicarbonate solution containing 1 m-mole glucose and 200 μmoles each of L-aspartic and α-oxoglutaric acids. (Mean values for duplicate experiments)

Perfusion time (min)	Glutamic acid produced during perfusion (μmoles)	Oxaloacetic acid produced during perfusion (μmoles)
60	36	37
90	48	48
105	52	52
120	58	54

120 min, was determined from the rate of glutamic-acid formation on subsequent incubation of the perfusates with aspartic and α-oxoglutaric acids. The transaminase activity in these perfusates (total vol. 60 ml.) calculated from the initial rate of reaction, was 42 μmoles/hr, which gives a Q_T of 6 (for hearts weighing 150 mg), less than half the activity observed if the substrates are circulated through the heart. There was little variation of this extractable transaminase activity with different times of perfusion and it seems unlikely that the entire glutamic/aspartic transaminase activity observed in hearts perfused with substrates in the circulating medium was the result of enzyme diffusing into the perfusion fluid. The extractable transaminase activity was much less than that found some hours after acute heart damage (Agress *et al.* 1955) and was of the same order as that observed in normal human serum (1 μmole glutamic acid formed/ml.hr) by LaDue & Wroblewski (1955).

DISCUSSION

Previous workers have shown that during perfusion of the isolated rat heart, amino acids added to the perfusate are metabolized to only a small extent. For example, Clarke (1957) found that less than 5 % of the total respiratory CO_2 was derived from amino acids added to the perfusate, and Clark *et al.* (1931, 1938) and Cruickshank & McClure (1936) found no

evidence of ammonia formation. The experiments reported above are in agreement with these findings, for not only was glutamic acid not utilized when added alone to the perfusate, but the glutamic/aspartic transaminase activity was only 3–4 % of that observed by Cohen & Hekhuiss (1941) in rat heart homogenates, and no other transaminase activity was detected. The inability to demonstrate any activity of the large number of transaminases which Cammarata & Cohen (1950) showed to be present in rat heart muscle was not due to inability of the acids to penetrate the heart cells, for the heart content of aspartic acid, leucine and alanine increased after perfusion with fluid containing these acids, and of the keto acids pyruvic at least must enter the cells since Clarke (1957) has shown that it is oxidized by the perfused heart.

At least a part of the glutamic/aspartic transaminase activity observed during perfusion was due to enzyme released into the perfusate, and it is possible that this enzyme is concerned with the maintenance of a high concentration of glutamic acid within the heart cells. It is of interest that the glutamic acid concentration in the rat heart (100–140 mg/100 g wet wt.) is nearly as high as that found by Waelsh (1951) in rat brain (153 mg/100 g), and much higher than that which he found in rat skeletal muscle (18–20 mg/100 g). Christensen (1955) has suggested that the mechanism of transport of glutamic and aspartic acids into cells differs from that of other amino acids and may involve transamination, and Wiseman and his co-workers (Matthews & Wiseman, 1953; Neame & Wiseman, 1957, 1958) have found that transamination occurs during absorption of glutamic and aspartic acids across the small intestine. Whether or not the glutamic/aspartic transaminase has this function in cardiac muscle it seems certain that, at least in the perfused heart, it is not concerned with energy metabolism.

SUMMARY

1. Reversible transamination has been demonstrated in the isolated perfused rat heart between aspartic and glutamic acids but not between other pairs of amino and keto acids.

2. The activity of the glutamic/aspartic transaminase was much less, $Q_T = 15$, than that previously demonstrated in homogenates of heart muscle.

3. Some transaminase was released into the perfusate.

4. There was no appreciable utilization of glutamic acid added alone to the perfusate nor of oxaloacetic acid produced during perfusion.

5. Alanine, leucine and aspartic acids, when present in the perfusate, penetrated to the heart cells but the glutamic-acid content of the heart, already high, was not increased when this acid was included in the perfusate.

We are indebted to Professor Fisher and Dr Zachariah for permission to quote their unpublished values for extracellular heart spaces. We should also like to thank Mr J. A. T. Saul for help with some of the estimations and the Medical Research Council and Imperial Chemical Industries Fellowship Committee, respectively, for the award to one of us (R. M. H.) of a research training studentship and a fellowship.

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POTENTIAL FIELDS INITIATED DURING MONOSYNAPTIC ACTIVATION OF FROG MOTONEURONES

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It has been noted that the patterns of discharge evoked in frog spinal motoneurones are significantly different when a presynaptic volley is initiated in dorsal root fibres and when the excitation arrives over a system of fibres in the lateral column (Brookhart, Machne & Fadiga, 1959). Anatomical studies (Liu & Chambers, 1957) raise the possibility that the differences may be ascribed to variations in the site of synaptic terminations on the motoneurones or to differences in the number of interneurones involved in the two pathways, or both. The present study was undertaken in order to determine which of these possibilities is the more likely. The technique of focal recording from within the spinal cord, and comparison of the temporal relations of simplified responses, have permitted a test of the hypothesis suggested by anatomical findings.

METHODS

The experiments have been performed on isolated spinal cords of frogs (*Rana pipiens*) during winter and spring months. The arrangement has been similar to that described previously (Brookhart *et al.* 1959; Machne, Fadiga & Brookhart, 1959). Micropipettes of 5–15 MΩ resistance, filled with potassium citrate, were used for depth recording. Ipsilateral evoked focal potentials were recorded unipolarly through an impedance-matching preamplifier, a high-gain, RC-coupled amplifier (time constant 1 sec), and photographed from an oscilloscope screen. A second oscilloscope was usually used to monitor ipsilateral ventral root responses. A thermistor thermometer in contact with the cord made possible temperature control in the range from 15–17° C.

The method of preserving function in isolated dorsal and ventral roots was important to the success of these experiments. The root was extended laterally on a small slip of filter paper coated with thinned petroleum jelly. The root was laid over hooked platinum electrodes and a complete covering of petroleum jelly delivered from a syringe and hypodermic needle was built around the entire root. This procedure not only preserved the fibres of the root, but also prevented troublesome electrical instability and provided the necessary insulation of the ventral root from the fluid surrounding the cord. In recording from the ventral root, the distance between proximal lead and cord varied in different experiments from 3 to 5 mm; the distal lead was always applied as far as possible from the cord on the crushed end of the root.

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Penetrations of micro-electrodes required that the pia should be dissected after tryptic digestion (Machne *et al.* 1959). The cord was oriented beneath the micromanipulator to permit penetration at various angles starting from the ventral or lateral surface, depending on the purpose of the specific experiment. Records were taken at intervals of 100 μ. To facilitate identification and measurement of the electrode tracks, after the last penetration the cords were usually hardened with formalin for 2 hr before removal of the micro-electrode. Shrinkage of the stained cross-sections was estimated from measurements of anteroposterior and lateral diameters of the live cord and from the ratio of micrometer reading to track depth in the prepared section.

In most of these experiments it was desirable to depress neuronal responsiveness. This was achieved through the addition of pentobarbital sodium (1:5000) to the bathing fluid (Eccles, 1946). Two reservoirs of Ringer's fluid were available, one of which contained the pentobarbital, connected to the recording chamber with a two-way stopcock. The intensity of pentobarbital effect was monitored by using the ventral root response to both routes of excitation. Usually no less than 30 min of continuous administration were required to reach the desired depth of depression. At this time normal Ringer's fluid was allowed again into the chamber and observations were initiated. The use of the barbiturate was resumed as soon as the threshold of ventral root responses began to shift toward lower values. Such a procedure was necessary in order to keep the level of depression fairly constant. Continuous administration of pentobarbital for more than the indicated time generally caused the preparation to undergo irreversible damage. Lower concentrations of pentobarbital with continuous administration were tried in a few experiments, but suitable and stable levels of depression were never achieved.

Bernstein & Weatherall (1952) has been used as a guide in the treatment of data.

RESULTS

In the absence of drug depression, stimulation of motoneurones over dorsal root (DR) or lateral column (LC) fibres gives rise to different patterns of discharge (Brookhart *et al.* 1959). In addition, focal potentials recorded from the dorsal and ventral horns also show striking differences. These differences are illustrated in Fig. 1. Focal potentials initiated in the ventral and dorsal horns upon DR and LC stimulation were recorded simultaneously with responses in the ventral root (VR). For preliminary comparisons, the beginning of the VR response may be used as an index of the onset of post-synaptic events in the focal recording. The post-synaptic activity recorded from the ventral horn yielded recordings which were initially negative following LC stimulation and initially positive following DR stimulation. Conversely, in the dorsal horn, post-synaptic activity is accompanied by a small positive-going slow wave following LC stimulation and an initially negative response following DR stimulation. These changes permit the inference that DR excitation initiates post-synaptic activity first in the dorsal horn, and only later does the motoneurone pool become active. LC stimulation initiates post-synaptic responses in the vicinity of the motoneurone pool and signs of activity do not appear in the dorsal horn.

Thus, this sort of study supports, in a preliminary fashion, the hypothesis based upon anatomical information. On the other hand, it does not

add information concerning the site of generation of the responses within the motoneurones. Specific signs of dendritic responses of motoneurones would be masked, in the dorsal horn (Fig. 1 *D*), by the activation of internuncial neurones which might, in turn, contribute terminals to any part of the motoneurone. Activity of internuncials would also mask events produced by whatever direct, monosynaptic connexions might exist

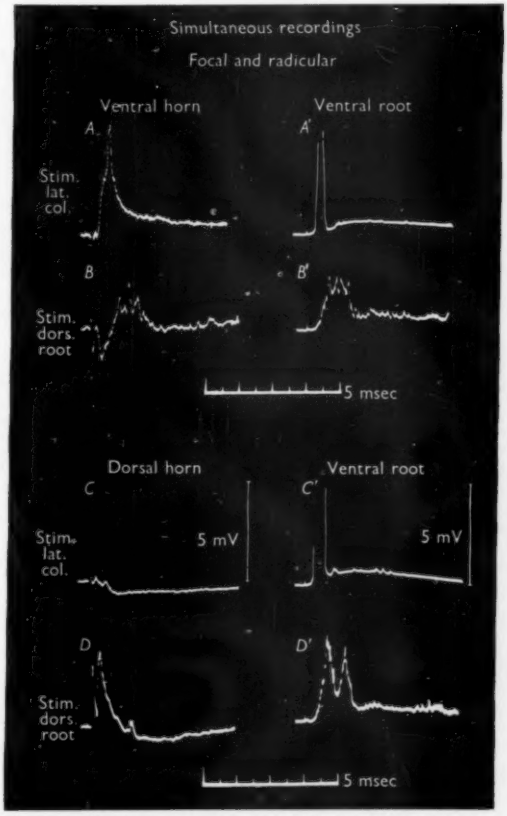

Fig. 1. Focal and radicular responses recorded simultaneously in a single untreated preparation. Focal responses from the ventral horn (*A* and *B*) and dorsal horn (*C* and *D*) were initiated by two routes of stimulation as indicated to the left. Note the opposite phases of focal post-synaptic components associated with the two forms of stimulation. To the right, the patterns of discharge in ventral root fibres. Part of the highly synchronized discharge initiated by lateral column stimulation is off-screen. Note the pattern of after-discharge. In this and all subsequent recordings, negativity at the active electrode is recorded by an upward deflexion. The spikes recorded from ventral root fibres (*A'* and *C'*) have been retouched to restore losses in photography.

between DR terminals and those dendritic expansions of motoneurones whose existence in the dorsal horn has been histologically proved (Sala, y Pons, 1892; Liu & Chambers, 1957). Older anatomical studies (Van Gehuchten, 1892) led to the conclusion that such monosynaptic connexions do not occur in Amphibia. Nevertheless, some of the results described in the physiological literature on the frog's spinal cord would be better explained by assuming the operation of a two-neurone reflex arc (cf. Barron & Matthews, 1938; Eccles, 1946; Marx, 1950; Fuortes, 1951). Studies of intracellular potential changes reported in a previous paper (Machne *et al.* 1959) have called attention to this possibility.

Experimental procedure

The experimental situation has therefore been simplified, and a procedure has been utilized in which all post-synaptic discharges were eliminated. The effects of applied stimuli were consequently confined to those structures directly impinged upon by primary fibres. It is generally agreed that electrotonic propagation does not take place across synaptic junctions, with the exception of certain structures, characterized by special geometric arrangements (Furshpan & Potter, 1959; Bullock & Hagiwara, 1957; Bullock, 1953). Since ventral root slow waves are electrotonically propagated, they must reflect EPSP's of motoneurones exclusively. If motoneuronal EPSP's are generated by primary terminals impinging on different portions of the neurone, the study of the distribution of focal signs of post-synaptic activity associated with ventral root slow wave should reveal the location of the activated portions of the motoneuronal membrane. The validity of this reasoning (Eccles, 1946; Brooks & Eccles, 1947; Eccles, Fatt, Landgren & Winsbury, 1954) is dependent upon the actual and complete suppression of every post-synaptic discharge, so that only stationary potentials are initiated in post-synaptic elements. As a consequence, internuncial relays must be blocked and volleys initiated in DR and LC fibres must evoke nothing but elemental VR slow waves.

Such a situation has been attained by the use of deep depression with pentobarbital and adequately weak stimuli. As previously noticed by Eccles and co-workers, it was often impossible, within workable levels of depression, to block every polysynaptic pathway. To overcome this limitation, a careful adjustment of the intensity of stimulation was required, until the voltage was found which would evoke only VR slow waves of elemental time course. Figure 2 gives direct evidence of the reliability of slow wave contours as an index of the synchronous response of motoneurones to a presynaptic volley which has not been temporally dispersed by internuncial paths of different lengths. A series of responses is reproduced, evoked in a depressed cord by DR stimuli of different

intensities and recorded simultaneously from dorsal horn and from ventral root. Although no stimulus ever evoked motoneuronal discharge, yet the time course of the slow wave did not become elemental as long as post-synaptic, asynchronous discharges appeared in the dorsal horn tracings. Subliminal motoneuronal re-excitation also occurred following LC stimulation if the intensity was too great or if the stimulating electrode was not adequately located in or on the lateral column. This location proved to be highly selective; minimal displacement from the optimal point (found by trial and error) readily changed the time course of recorded slow waves.

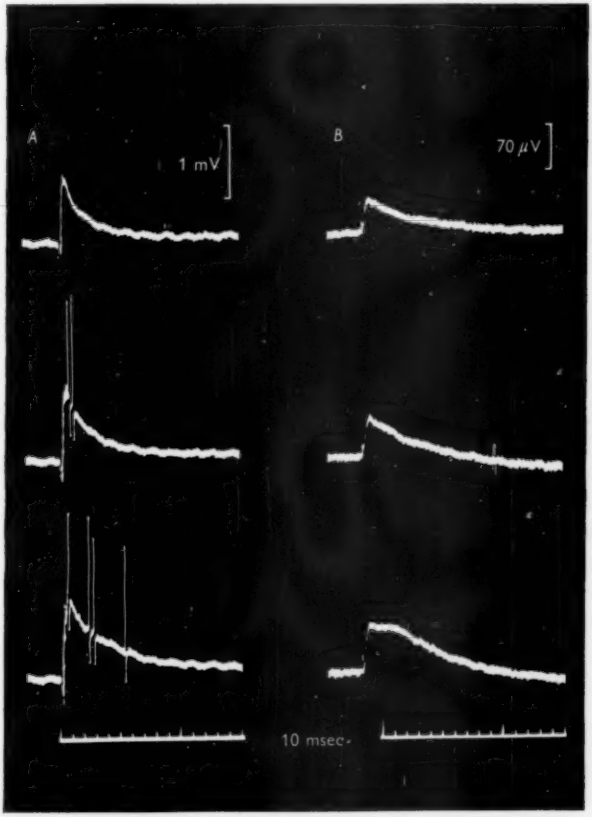

Fig. 2. Focal and radicular responses recorded simultaneously in a preparation treated with pentobarbital. Dorsal root stimuli of increasing intensity from top to bottom. With the exception of the top recordings, the dorsal horn focus (*A*) shows increasing interneuronal discharge which correlates with characteristic departure from exponentiality in the decay phase of the ventral root slow wave (*B*). The spikes recorded from the dorsal focus have been retouched to restore losses in photography.

Ventral root responses showed a different sensitivity to pentobarbital depression, depending upon the route of activation. As a rule, after 20–30 min of perfusion with pentobarbital-Ringer's solution even maximal DR stimuli failed to evoke motoneuronal discharges, thus confirming Eccles's earlier results (Eccles, 1946). The synchronized outburst of short latency evoked by LC stimulation in the untreated cord (Brookhart *et al.* 1959), on the other hand, diminished only slightly in amplitude until the general depression of the preparation became irreversible. This different sensitivity enabled us to prevent any undue impairment of motoneuronal excitability. The maximal response elicitable from VR upon LC stimulation was periodically checked: no observations were made if it became impossible to evoke the short-latency, highly synchronized discharge. If not specified otherwise, the actual observations were carried out with stimuli so adjusted as to evoke, for both ways of activation, the maximal slow wave still maintaining an elemental time course.

Experiments have been performed in the above-defined conditions in order (*a*) to identify the nature of potential changes focally recorded; (*b*) to map their distribution within the spinal cord and (*c*) to study the temporal relationships between such potentials and VR-recorded slow waves.

Nature of focal responses. Evoked potentials recorded from specific foci upon LC or DR fibre activation and obtained by stimuli of increasing voltage are shown in Fig. 3. Identification of the various components of the responses is provided by the close similarity to reactions obtained from the cat spinal cord under comparable conditions (Brooks & Eccles, 1947). The evoked potential change begins with the diphasic signs of the presynaptic volley, approaching and eventually reaching presynaptic terminals. This spike is followed by the post-synaptic response, characterized by a time course fast in its rising phase and slower in its decay, which appears to be exponential. Such evolution is apparent in all tracings reproduced in Fig. 3, and does not change with varying intensity of stimulation. This is proof of the elemental, graded nature of the recorded post-synaptic reactions. The time course of the decaying phase of the post-synaptic reactions is illustrated in more detail by the plotted values in Fig. 4. The apparent exponential character of the decay is confirmed by the close approach to linearity exhibited by these semi-logarithmic plots. The slight departure from linearity, particularly noticeable in the responses to LC excitation, has possible implications which cannot be dealt with at this time (cf. Fatt, 1957*b*). Since stimuli evoking these focal responses also elicited slow waves of elemental time course in ventral roots, the actual existence in the frog of monosynaptic connexions between motoneurones and both routes of activation can be considered as proved.

Accurate measurements of parameters of focal potentials were performed in a number of experiments, in order to compare the responses set up by LC or DR stimuli. The results of many observations are summarized below in terms of the mean value and standard error. Conduction time, measured by the interval between stimulus artifact and peak of the positive phase of presynaptic component, was $0{\cdot}45 \pm 0{\cdot}057$ msec (n = 9) and $0{\cdot}61 \pm 0{\cdot}052$ msec (n = 15) respectively for LC and DR stimulation. The interval between the arrival of the presynaptic volley at the recording point and the onset of the post-synaptic potential, expressing the duration of synaptic delay, averaged $1{\cdot}10 \pm 0{\cdot}075$ msec (n = 17) (LC stimulation) and

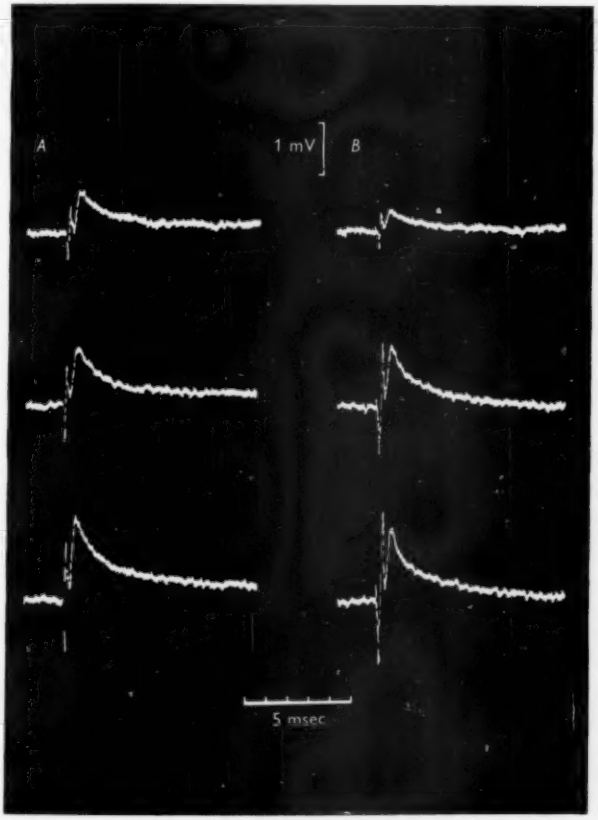

Fig. 3. Focal responses to increasing intensities of stimulation. *A*, dorsal horn responses to DR stimuli. *B*, ventral horn responses to LC stimuli. In all recordings the fast diphasic portion of the complex is presynaptic in origin. Note the increase in amplitude without change in time course of the post-synaptic portions of these complexes.

$1{\cdot}40 \pm 0{\cdot}083$ msec (n = 15) (DR stimulation). Both differences are significant ($P < 0{\cdot}02$). The mean duration of the rising phase of post-synaptic potentials was practically the same in the two cases, equalling $1{\cdot}65 \pm 0{\cdot}092$ (n = 17) (LC stimulation) and $1{\cdot}67 \pm 0{\cdot}081$ msec (n = 13) (DR stimulation). Likewise no significant difference was found in the time constant of exponential decay. For LC-evoked and DR-evoked responses

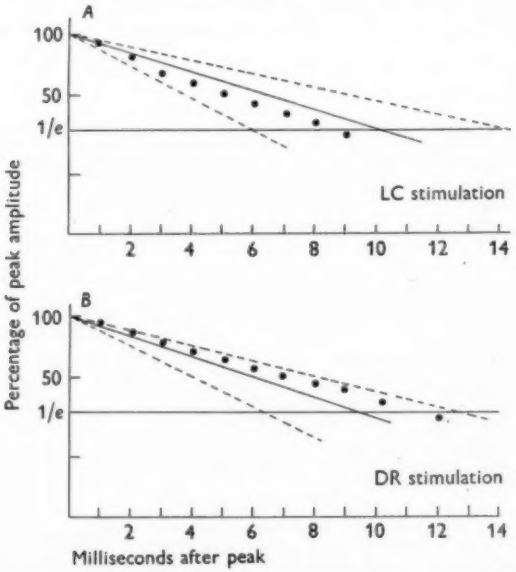

Fig. 4. Decay phases of post-synaptic responses plotted on semilogarithmic coordinates. Responses to lateral column stimulation (*A*) recorded from ventral horn; responses to dorsal root stimulation (*B*) recorded from dorsal horn. The heavy lines indicate the mean values obtained from eight preparations; the dashes include two standard deviations; the plotted points represent characteristic single preparations not included in the means. The time constant of the exponential decay is given by the intersection with the $1/e$ ordinate. The difference in time constant of decay for the two focal potentials is not significant.

the mean values were $10{\cdot}1 \pm 1{\cdot}46$ (n = 8) and $9{\cdot}5 \pm 1{\cdot}14$ msec (n = 8); the probability that this small difference had occurred by chance in the treated sample is more than 60 %. To summarize, it can be said that only the parameters of the presynaptic components depended upon the route of stimulation; the evolution of post-synaptic potentials can be considered elemental and identical in the two cases.

The longer conduction time measured in DR-evoked focal potentials is explained by the technical arrangement used in these experiments. Between focal probe and DR-stimulating electrode the distance was greater than

that between focal probe and LC-stimulating electrode. As a matter of fact, differences in conduction time as great as half a millisecond were observed in some cases (see Fig. 8) in which the placement of DR-electrode was unusually far from the root entrance; for this reason they have not been included in the mean. On the other hand, the synaptic delay was also longer in DR-evoked than in LC-evoked focal potentials. Since the time course of post-synaptic components was virtually independent of the route of activation, it seems reasonable to refer this difference to characteristics of presynaptic terminals.

All the values quoted above are slightly greater than those measured in monosynaptically induced focal potentials recorded from the cat's spinal cord. However, the two ranges are comparable in magnitude. The small differences can be accounted for by diversity of species, since their sign agrees with the general pattern shown by comparison of homologous phenomena in Mammalia and Amphibia.

Topographical distribution of focal potentials. Although the time course of post-synaptic potential changes was the same for both forms of excitation, studies of their distribution throughout the cross-section of the spinal cord revealed clear differences. In the maps of Fig. 5, areas bordered by the heavy solid lines show the average distribution of negative focal post-synaptic potentials as described above, 20% of the maximal amplitude being chosen as marginal value (cf. Eccles *et al.* 1954). (For the sake of brevity, hereafter the expressions 'focal post-synaptic potential' or 'focal post-synaptic response' if used without any specification of polarity shall be meant to indicate only negative-going potential changes.)

The patterns of distribution observed upon DR and LC stimulation differed greatly, and no overlapping was seen to occur between the two activated areas. Stimulation of DR activated a region mostly confined to the dorsal horn, but post-synaptic potentials evoked by LC stimulation were detected from the ventral horn and from the ventromedial funiculus. Within these regions, the amplitude of the post-synaptic response was not uniform. The areas of major potential change are indicated by the cross-hatching in Fig. 5. It should be noted that the two most dorsal foci activated by DR fibres are in regions where, in addition to internuncial elements, rich dendritic arborizations from motoneurones have been histologically described (Sala y Pons, 1892). The major focus closely corresponding with the motoneurone pool is specifically activated by LC stimulation. The focus in the intermediate zone is characterized anatomically by predominance of internuncial cells. The smaller foci indicated for LC stimulation along the ventromedial margin of the ventral horn were not constantly found. Their positions, moreover, varied markedly in successive experiments. We suspect that one of the sources of this variability might

reside in small differences in the placement of stimulating electrodes. Detailed consideration of the variation of response within the areas of focal negativity will be presented below.

It has seemed of interest to establish the location of the sources of current caused to flow by focal post-synaptic potentials. If the focal post-synaptic potentials originate primarily from motoneurone activation, the distribution of related sources should conform to the anatomical distribution of those portions of the motoneurone membrane which have not

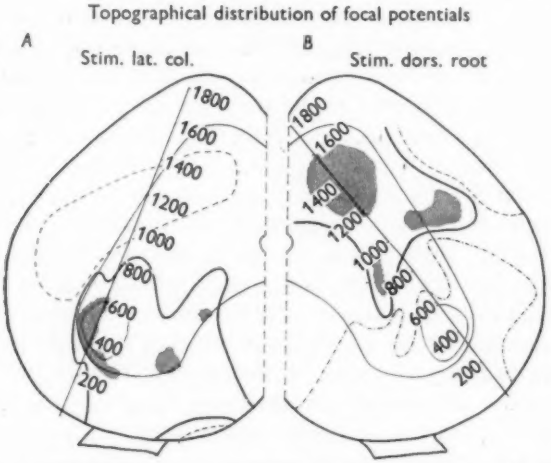

Fig. 5. Distribution of focal responses to two forms of stimulation. A schematized cross-section of segment X is presented. The circles in the ventral horns outline the borders of the motoneurone nucleus. Foci related to lateral column stimulation are indicated on the left; those related to dorsal root stimulation on the right. The heavy lines delimit the areas in which focal negativity was recorded. The cross-hatched areas represent zones of particularly intense focal negativity in many experiments. The interrupted lines delimit zones in which positive-going waves occurred coincidentally with post-synaptic focal negativity in other areas, dot-and-dash lines indicating boundaries better defined than those marked by dashes only. The straight lines describe the paths traversed by electrodes during the recording of responses illustrated in Fig. 6. The calibrations are depths beneath point of entry of penetrating electrode. Note the complete lack of overlap of zones of focal negativity initiated by the two forms of stimulation.

been directly activated. Judging from the distribution of positive-going waves, it would appear that activity initiated by LC terminals on motoneurone bodies and proximal dendrites draws currents from axons in the ventral white matter and from terminal dendrites lying dorsally; synaptic activity initiated in distal portions of the dendritic tree by activation of DR fibres is chiefly associated with sources in motoneurone bodies and

axons in the ventral regions of the spinal cord. Such a distribution would be highly unlikely if the focal post-synaptic potentials originated solely from interneuronal activation. In the maps of Fig. 5, the interrupted lines define regions from which positive-going potential changes indicative of sources have been recorded. Both for LC and DR stimulation, the over-all distribution suggests that the production of focal post-synaptic response was due, at least in part, to the activation of motoneurone membrane. Upon LC stimulation the boundaries were sometimes difficult to define and varied somewhat from one experiment to another; moreover, the amplitude of recorded potentials was small. In some experiments no positive potentials at all were recorded upon LC stimulation. Possible explanation for this behaviour is provided in the Discussion.

Details of the changes of configuration of post-synaptic responses are illustrated in Fig. 6. In this figure two typical series of responses are arranged according to the depth of an electrode tip passing through the motor nucleus from its ventrolateral aspect to penetrate the region of internuncial and motoneuronal dendrites in the dorsal horn. One of these series was recorded during LC stimulation and penetration along the track in the map of Fig. 5*A*; the other was recorded during DR stimulation and penetration along the track indicated in Fig. 5*B*.

It can be seen that LC-evoked focal post-synaptic potentials became progressively larger as the electrode approached and progressively smaller upon leaving the motoneurone pool; eventually they were replaced by a positive-going response. The voltage change occurring in this dorsal source was very small; its time course was roughly comparable to the time course of the associated focal post-synaptic potentials. The reversal either happened through a slightly diphasic response, as can be seen in the series reproduced, or involved the passage across a zone of equal potential with the reference electrode. No clear correlation has been established between location of reversal zone and reversal pattern.

Upon DR stimulation the changes occurring in focal responses with progressive penetration were approximately the opposite to those observed with LC stimulation. Focal excitatory post-synaptic activity was recorded from the deep regions of the dorsal horn, while in correspondence with the motoneurone pool, and more widely throughout the ventral horn, a positive-going event was recorded. Examination of tracings obtained with faster sweep speeds permitted a precise determination of the relationships between time course of dorsal sink and time course of source recorded from the ventral portions of the track. A sharp identity of latencies and times to peak contrasted with a difference in the decay phases. The decay of the ventral positivity was much faster than that of the dorsal focal post-synaptic potential; moreover, it could not be fitted on a straight line if

plotted as logarithm of potential against time. The reversal between dorsal negativities and positive-going deflexions involved, as a rule, the passage through a zone of diphasicity, in which the post-synaptic response began with a short positive deflexion, followed by a longer-lasting negative wave. It will appear from the Discussion that both this reversal pattern, and the lack of correspondence between time course of sources and sinks, could be explained by the operation of a distorting factor in ventral regions of the cord when post-synaptic activity was developing in the dorsal horn.

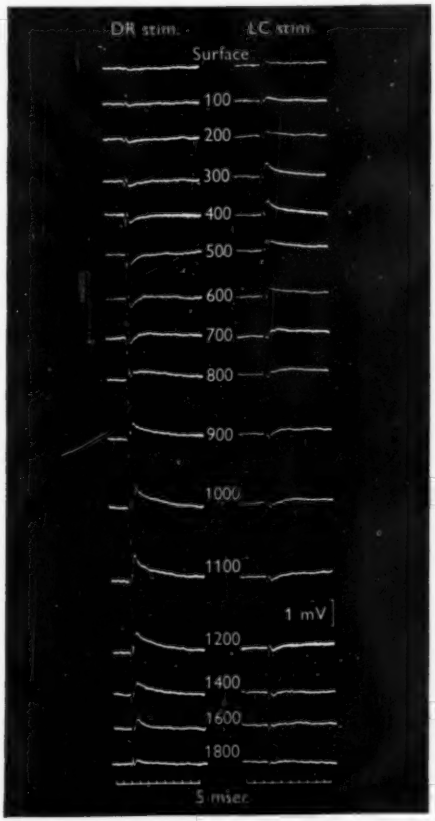

Fig. 6. Changes in configuration of focal potentials during penetration to successively greater depths. Potentials initiated by DR stimulation in column to left; those initiated by LC stimulation in column to right. The calibrated electrode tracks are indicated in Fig. 5. Note the distribution of post-synaptic negativity in the more dorsal portions of the penetration following DR stimulation and in the ventral horn zones following LC stimulation.

Gradients within focal areas. It has already been stated that the amplitude of the post-synaptic response varied in different portions of a focal area of negativity. On the assumption that areas of concentration of synaptic knobs would give rise to maximal responses within a focus, it seemed worth while to examine amplitude changes in more detail. Careful

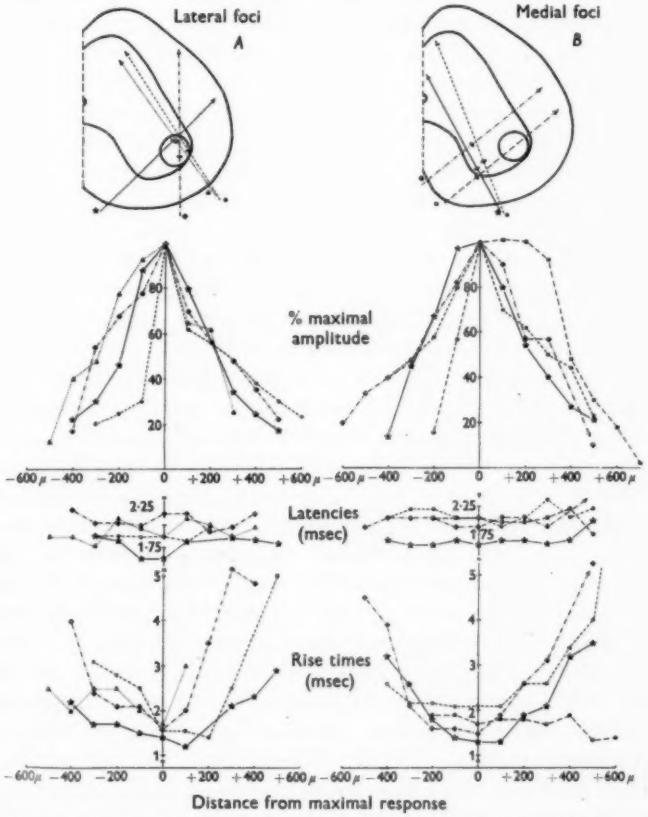

Fig. 7. Alterations of focal potentials within fields activated by lateral column stimuli. The measurements deal with foci which were maximal in the region of the motoneurone nucleus (*A*) and in the medial portion of the ventral horn among internuncials and motoneurone dendrites (*B*). The small maps at the top each illustrate the courses of four penetrations. On each track, the point of maximum response amplitude is indicated by a small heavy cross-bar and relates to the zero abscissal value in the graphs below. The relation between each line-graph and the proper electrode track is indicated by the symbols and line characteristics. The uniformity of latency, coupled with concurrent decrease in amplitude and increase in rise time, suggests that synaptic activation occurs in a restricted focus and that negativity spreads from this focus by electrotonic propagation.

inspection of the records revealed that, in addition to amplitude gradients, the responses within a focal area of negativity were also characterized by progressive changes in time course.

The results of studies of the responses due to lateral column stimulation are presented in graphic form in Fig. 7. The families of curves shown in this figure refer to tracks coursing in various directions across the ventral horn. Directions of various tracks as determined from histological preparations are shown in the diagrams above the graphs. Percentage changes in peak amplitude, duration of rising phase, and latency, have been plotted as a function of distance from that point along each track which yielded the maximal response.

The data utilized for the graphs on the left (Fig. 7*A*) refer to tracks passing through the motor nucleus. One can see that in all these tracks the locus of maximal response fell within the limits of the motoneurone pool. With increasing distance from the pool, the amplitude of post-synaptic response decreased rapidly, and this decay was regularly linked with lengthening time to peak. The latency, however, remained practically constant throughout the whole series of records.

The four penetrations yielding the data displayed in the graphs of Fig. 7*B* crossed the medial portion of the ventral horn. In each of these penetrations the maximal response was recorded from a focus outside the motoneurone pool. With one exception, the gradients of amplitude and time course were essentially the same as those noted for penetrations which did traverse the motor nucleus. Again, no significant changes in latency were noted. In the exceptional case, the tip of the electrode passed through both the ventromedial field and the motor nucleus. The responses recorded during this penetration failed to show any decrement over approximately 400 μ, until the tip of the electrode passed the middle of the motor nucleus. The inter-individual variation in positions of foci in the ventromedial area, which has been mentioned previously, is indicated by the scatter of maximal points on the tracks of Fig. 7*B*. In some experiments, no evidence of a medial focus was found (see track '★', Fig. 7*A*).

A similar analysis, carried on for data obtained upon DR stimulation, showed that in the dorsal horn focal post-synaptic potentials remained at a nearly constant amplitude over distances longer than those measured in the ventral horn for LC-evoked responses. The decrement observed while leaving areas of maximal response was not accompanied by proportional delay in rise time until the micro-electrode was very close to the boundaries of the region from which negative monophasic responses could be recorded.

The observations on ventral horn potentials thus reveal a close correlation between amplitude gradient of post-synaptic response and alterations in rise time, whereas latencies are quite independent of the electrode

position. If it is assumed that variations in synaptic density are the only cause of variations in amplitude, the observed alterations in rise time would not be predicted. Considering the uniformity of latency, the more reasonable explanation of both amplitude and temporal gradients is that the focal negativity outside the area of maximal response results from electrotonic propagation into inactive areas from directly activated subsynaptic zones on the same membrane. The lack of a clearly recognizable exponential rate of decrement does not constitute critical evidence against this interpretation. Exponential decrement might be masked or distorted while recording unipolarly from a volume conductor in which structures of different size, length and orientation are simultaneously activated. It would seem, therefore, that LC stimulation results in activation of synaptic terminals in rather sharply restricted zones within the ventral horn. Concerning the one exceptional case in Fig. 7*B*, in which amplitude decrement and temporal distortion did not occur in such a way as to suggest a restricted zone of activation, it is known that long motoneurone dendrites mingle with internuncials in the medial portion of the grey matter (Sala y Pons, 1892). It has also been shown that lateral column fibres terminate in this same region (Liu & Chambers, 1957). Consequently, both pre- and post-synaptic elements necessary for distributed, as contrasted to focal, activation are present. In the dorsal horn, likewise, the lack of proportionality between amplitude decrement and prolongation of rise time suggests that synaptic activity is initiated by DR stimulation over a wide field within the dorsal horn. Synaptic connexions are therefore likely to be made with most of those structures whose presence has been histologically demonstrated within the region from which focal post-synaptic negativity can be recorded upon DR stimulation.

Temporal relationships between focal post-synaptic potentials and ventral root slow waves. It has already been pointed out in a previous section that under the conditions of these experiments focal post-synaptic potentials elicited upon stimulation of LC and DR fibres must originate from elements activated by terminals of primary fibres. Since stimuli used in evoking such potentials were effective in eliciting elemental slow waves in ventral roots, the existence of a continuous membrane between activated zone and motor axons can be safely inferred. The total lack of overlap between distribution of focal post-synaptic potentials set up over the two routes of stimulation forces the conclusion that DR fibres and LC fibres establish such connexions in different sites on motoneuronal membranes; that is, with the long dendritic expansions stretching into the dorsal horn in one case, and with cell bodies and possibly most proximal dendrites in the other. This conclusion has been submitted to crucial test by analysing the temporal relationships between focal potentials and associated VR slow

waves. If the interpretation is correct, the consequence of the greater distance covered by DR-evoked slow wave in its electrotonic propagation from the dorsal horn must become apparent in the form of a greater difference between the time course of the focal potential and the resultant VR slow wave. Since, in the frog, the motoneurones are tightly clustered in a narrow cell column, unequal axonal lengths need not be considered as a possible source of variations.

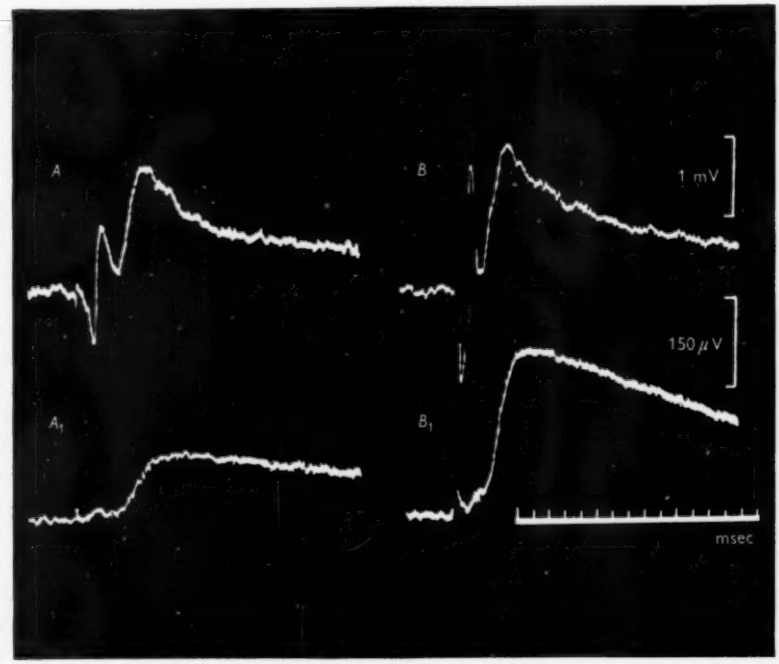

Fig. 8. Comparison of time course of focal and radicular responses to two forms of excitation. All the tracings refer to the same preparation. In *A* the dorsal horn focal response was recorded simultaneously with the VR slow wave (A_1) following DR excitation. In *B* the ventral horn focal response was recorded simultaneously with the VR slow wave (B_1) following LC excitation. Although each radicular response has a latency identical with that of the related focal post-synaptic response, the rise and decay times are distorted. The degree of distortion is greater for DR responses than for LC responses.

Examples of records from which the critical data have been drawn are presented in Fig. 8. Simultaneous records of the appropriate focal response and the associated VR slow wave illustrate the events following stimulation over the two routes in the same animal. It is immediately apparent that the onset of the post-synaptic events is simultaneous in each pair of

records. It is also apparent that the rise and decay times of the post-synaptic waves are longer in the ventral root recordings than in the focal recordings. Careful measurements reveal that the rise times of both focal potentials are essentially the same (DR, 1·85 msec; LC, 1·70 msec). Furthermore, the prolongation of the rise time of the ventral root response is greater following DR excitation than following lateral column excitation (DR, 3·70–1·85 = 1·95 msec; LC, 2·65–1·70 = 0·95 msec). Similar measurements have been carried out on records from a number of experiments which permitted paired comparisons. All the measurements presented in Table 1 were derived from ten individuals. In each individual, both forms of response were elicited from the same spinal cord and differences were evaluated by comparisons of the pairs of measurements. For both routes of stimulation, the correspondence of the latencies of focal and VR potentials is so close as to obviate the necessity for statistical validation.

TABLE 1. Latencies and rise times of post-synaptic events initiated by two routes of stimulation and recorded from appropriate foci and ventral roots

(Mean ± S.E.; n = 10; paired values.)

	DR stimulation		LC stimulation	
	Latency (msec)	Rise time (msec)	Latency (msec)	Rise time (msec)
Focal post-synaptic potential	1·88 ± 0·143 (*a*)	1·67 ± 0·092	1·58 ± 0·132 (*a*)	1·65 ± 0·120
Ventral root slow wave	1·93 ± 0·095 (*b*)	4·10 ± 0·251 (*c*)	1·69 ± 0·133 (*b*)	3·20 ± 0·293 (*c*)

Mean differences and their statistical significance

(*a*) Focal latency: mean (DR minus LC) = +0·297 ± 0·135; $t = 2·200$; $0·10 > P > 0·05$.
(*b*) VR latency: mean (DR minus LC) = +0·245 ± 0·106; $t = 2·322$; $0·05 > P > 0·02$.
(*c*) VR rise time: mean (DR minus LC) = +0·900 ± 0·339; $t = 2·655$; $0·05 > P > 0·02$.

Similarly, the interval from initiation to peak of the focal post-synaptic response was the same for both foci. In contrast, the rise time of the ventral root slow wave was so much longer in both cases than that of the associated focal potential, that statistical validation was considered superfluous. Since the rise time of focal potentials was the same for both routes of excitation, the difference between the rise time of the two VR slow waves expresses an additional delay introduced into the VR response to DR excitation. The difference in rise time of ventral root slow waves has been statistically validated (see comparison '*c*', Table 1). Our results do not support the opinion (Bonnet & Bremer, 1952) that the distances between intraspinal foci and radicular recording point are not sufficiently great to produce a significant amount of delay.

Similar evidence of temporal distortion in the VR slow wave has been obtained by measurement of the time constant of decay. It will be recalled

that the time constant of exponential decay of each of the focal potentials was the same (Fig. 4). Careful measurements of decay curves of ventral root responses confirmed the prolongation which has been noted in the records of Fig. 8. In the sample of ten experiments included in Table 1, the mean time constant of decay was $33{\cdot}5 \pm 3{\cdot}99$ msec for LC-evoked slow waves and $38{\cdot}75 \pm 4{\cdot}58$ msec for DR-evoked slow waves. The differences between LC and DR decay time constants in the same individual had a mean of $5{\cdot}25 \pm 1{\cdot}957$. This mean difference is significantly different from zero ($t = 2{\cdot}683$; $0{\cdot}05 > P > 0{\cdot}02$). It is our opinion that the relatively large variances encountered arise primarily from the difficulty of establishing a single point on a slowly changing decay curve. Thus, the greater distortion of time course of the DR-evoked slow wave supports the interpretation of electrotonic propagation over a greater distance.

DISCUSSION

Inferences concerning the site of impact of the two kinds of presynaptic volleys on the motor neurones of the frog spinal cord may be derived from the following items of evidence. (1) The observations have been confined to focal and ventral root potential changes having a simple rising phase and an exponential decay. This time course attests the lack of internuncial discharge and the monosynaptic nature of the pathways under study. (2) The focal potentials initiated in the gray matter by the two forms of stimulation were identical in time course. Those evoked by LC excitation centred in the area occupied by motoneurones and proximal dendrites; those evoked by DR excitation centred in an area occupied by interneurones and dendritic extensions of motoneurones. (3) The onset of all focal potentials was simultaneous with the onset of the associated VR slow wave. (4) Comparison of the temporal characteristics of focal and radicular potentials revealed a distortion which was greater for responses evoked by DR stimulation than for responses evoked by LC stimulation. It is necessary to assume only that the post-synaptic membrane response is a stationary EPSP passively propagated to the ventral roots by electrotonus. The acceptability of this assumption has been well justified (Barron & Matthews, 1938; Eccles, 1946; Brooks & Eccles, 1947; Bonnet & Bremer, 1952). No other assumption would be consistent with the identity of latency between focal and radicular potentials. Within this framework lie all the elements necessary for the conclusion that LC activity influenced motoneurones over synapses located on cell bodies and proximal dendrites, whereas DR activity was delivered over primary fibres terminating on distal portions of the dendritic trees of the motoneurones.

Attention should be directed to certain simplifications adopted in the interpretation of the data. The potential changes dealt with have been

interpreted as excitatory post-synaptic potentials. Any inhibitory post-synaptic potentials of monosynaptic origin (Terzuolo, 1954; Gernandt & Terzuolo, 1955) must have been completely obscured by over-riding depolarization, since focal positivities in areas of active synaptic terminations have not been observed. Inhibitory post-synaptic potentials of di-synaptic origin would have been prevented by the internuncial blockade resulting from the pentobarbital. Focal positivity in the dorsal and ventral horns must be interpreted as source activity, since appropriate axon terminals do not exist in the areas from which positivity was recorded.

It is obvious that all post-synaptic elements activated by primary fibres would develop EPSP's and thus contribute to the focal potentials recorded from areas where interneuronal and motoneuronal membrane were both present. Inferences drawn from shifts in time course between focal and radicular potentials would be therefore valid only if both interneuronal and motoneuronal EPSP's have the same time course. Evidence that this is the case was obtained from the study of focal potentials evoked in the medial portion of the ventral horn outside the motor nucleus, where interneuronal elements are present in abundance. No differences in time course of focal potentials could be detected although the identity of the elements contributing to the response was different in the two positions (see Fig. 7*B*, track ■).

Because of the accelerating distortion inherent in the focal recording technique (Brooks & Eccles, 1947), the focal potentials studied must be somewhat faster than the EPSP's with which they are associated. Lack of knowledge of the true time course of the EPSP prevents comparison between the amounts of distortion observed after electrotonic propagation to the ventral roots and the amounts of distortion which could be predicted from known space and time constants. This is hardly important, since the error introduced by the focal recording technique must be the same for all responses. The radicular slow waves evoked by the two forms of stimulation are both produced by electrotonic propagation over continuous motoneuronal membranes. Differences in degree of alteration in time course induced by electrotonic propagation from two equal, uniformly distorted origins must be related to the portions of motoneuronal membrane not shared by the two responses. Since the cell bodies are so tightly clustered, the dendritic membrane is the only portion of the motoneurone which can be considered to be unique to the pathway of the DR response. Thus, alternative interpretations of the data summarized at the beginning of this section do not appear to be tenable.

Attention must now be directed to a consideration of the potential changes recorded outside the areas of focal negativity. It will be recalled that the response recorded from ventral horn regions following

DR stimulation is an initially positive (often diphasic) potential change and lies between a dorsal horn focus and a radicular response, both of which are initially negative in sign. Because of the gross differences between the time course of negativity in the dorsal horn and concurrent positivity in the ventral horn (see Fig. 6), the ventral horn positivity cannot be regarded simply as evidence of a ventral source furnishing current to a dorsal sink. It seems most reasonable to consider that the ventral horn acts as a source only until such time that current flows associated with electrotonically propagated negativity bring about an early decay of positive and often a reversal of potential sign at the tip of the electrode (cf. Lorente de Nó, 1947*a*; Fatt, 1957*a*). On this ground, diphasicity at the focal electrode would not constitute evidence of actively propagated events (Lorente de Nó, 1947*b*; Brooks & Eccles, 1947; Barakan, Downman & Eccles, 1949; Bishop & Clare, 1953). This explanation of the ventral horn response to DR excitation cannot be decisively admitted or discarded because it fails to take into account the current flows associated with interneuronal membranes whose anatomical distribution is unknown. However, intracellular recordings (Fadiga & Brookhart, 1960) show that DR excitation induces hypopolarization of the cell body at the same time that the surrounding medium becomes focally positive. Direct evidence is thus available that the contour of the ventral horn response to DR excitation is the result of interference between two events having opposite signs.

The low amplitude of the positive wave in the dorsal horn accompanying the ventral horn focal negativity evoked by LC stimulation was expected. The focal sink which develops in the region of the cell body can draw current from widely dispersed portions of the motoneuronal dendritic tree; thus the current density at any one focus in the source area should be quite small (Lorente de Nó, 1947*a*). The high gradients of electrotonic decrement which must occur in the thin terminal branches would minimize interfering current flows and allow the source activity to reflect with accuracy the time course of current flow toward the ventral sink. The contribution of current from axonal sources would be so small as to be difficult to detect (Fatt, 1957*b*). These predictions were all verified by the observations.

It still remains to be seen how the results of the present study correlate with the observations on ventral root discharges initiated by these two forms of stimulation in the absence of pentobarbital (Brookhart *et al.* 1959). There remains no doubt that the highly synchronized outburst elicited in VR by lateral column stimulation is the result of monosynaptic activation through axo-somatic synapses. In the absence of pentobarbital, this initial discharge is frequently followed by a low level of asynchronous after-discharge. It seems likely that this latter occurs as a result of reactivation of motoneurones through internuncial pathways coursing

through the medial portions of the ventral horn. Pentobarbital consistently abolished the after-discharge without seriously affecting the early, synchronized activity.

In the absence of pentobarbital, dorsal root excitation is followed by asynchronous motoneurone discharge which persists for 15–20 msec. In the presence of pentobarbital, monosynaptic activation of terminal dendrites consistently failed to produce motoneurone discharge. At the present time there is no basis for either accepting or rejecting the hypothesis that pentobarbital acts selectively to depress dendritic responsiveness more than it does that of the soma membrane. On the other hand, if it be assumed that pentobarbital depresses all portions of the motoneurone membrane to a similar degree, the failure of discharge is susceptible of two interpretations. Synaptic activation confined to distal portions of dendrites may be incapable of initiating firing because of the inability of dendrites to conduct propagated impulses (Clare & Bishop, 1955*a*, *b*) coupled with the decrement associated with electrotonic propagation over the long, thin dendritic branches. Under these circumstances, the dendritic response might not be able to depolarize the cell body to the firing level of its trigger zone (Coombs, Curtis & Eccles, 1957*a*, *b*; Fuortes, Frank & Becker, 1957; Eyzaguirre & Kuffler, 1955; Fatt, 1957*b*; Edwards & Ottoson, 1958). Alternatively, if dendrites are capable of propagating all-or-none responses (Cragg & Hamlyn, 1955), the lack of discharge may be due simply to an inadequate number or potency of synapses from dorsal root collaterals. In either case, if interneuronal firing is not prevented, interneuronal synapses in greater numbers on distal dendrites or applied nearer to the cell body would reinforce the response to monosynaptic dorsal root activation and result in motoneurone discharge.

Despite these uncertainties, the results of the present study afford evidence of a neuronal system which permits a clear separation between the effects of activation over synapses terminating on or near cell bodies and synapses terminating on distal portions of the dendritic trees of motoneurones.

SUMMARY

1. The isolated spinal cord of the frog has been utilized to obtain functional confirmation of suggested anatomical relations between motoneurones and cellulipetal fibres of the dorsal roots and of the lateral columns.

2. Functional depression produced by pentobarbital was used to eliminate interneuronal discharge. Under these conditions, it was demonstrated that monosynaptic activation of ipsilateral motoneurones may be brought about by stimulation of either the lateral column or the dorsal root. Such activation produces potential changes within the spinal cord

and in ipsilateral ventral roots in which presynaptic and post-synaptic components may be identified. The post-synaptic component may be recognized as elemental by virtue of its relatively brief, simple rising phase and by its exponential decay.

3. Upon focal recording the elemental post-synaptic response to dorsal root stimulation is confined to dorsal horn areas occupied by internuncial cell bodies and terminal dendrites of motoneurones. The comparable response to lateral column stimulation is confined to the ventral horn, being particularly prominent in the vicinity of the motor nucleus and occasionally in a medial area occupied by internuncial cells and motoneurone dendrites. In both dorsal and ventral foci the post-synaptic events have slightly different latencies but identical rise and decay times.

4. The ventral-root slow waves, originating by electrotonic propagation from the activated zones of the motoneurone membranes, have a significantly longer rise time and time constant of decay when excitation is initiated by dorsal-root fibres than when it arrives over lateral column fibres. Since the focal potentials have the same time course, and since the latency of the focal potentials is identical to the latency of the related ventral-root slow wave, the delayed rise and fall of the slow wave evoked by dorsal-root excitation must be due to the longer path over which spread of electrotonus occurs.

5. It is concluded that the spinal cord of the frog offers a neuronal system in which the results of terminal dendritic excitation can be clearly differentiated from the results of excitation at the cell body and proximal dendrites.

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THE CHEMICAL EXCITATION OF SPINAL NEURONES BY CERTAIN ACIDIC AMINO ACIDS

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The presence of comparatively large quantities of glutamic acid in nervous tissue has led to speculation concerning its function therein (Waelsch, 1955). Hitherto metabolic aspects have been stressed, but the recent demonstrations of the action of γ-amino-*n*-butyric acid (GABA) upon neurones (Curtis, Phillis & Watkins, 1959*b*) together with the metabolic relationship of this substance to glutamic acid (Waelsch, 1955) suggested that the actions of glutamic acid and related acidic amino acids upon neurones should be investigated. A preliminary report has been published (Curtis, Phillis & Watkins 1959*a*). The close similarity which has been observed between the action of cysteic acid and those of aspartic and glutamic acids has led to the inclusion of the sulphonic acid also in this report.

METHODS

The experimental procedure has been reported previously (Curtis *et al.* 1959*b*). The neurones were located in lumbar segments of cats lightly anaesthetized with pentobarbital sodium. Extracellular records were obtained by means of double and five-barrelled electrodes from interneurones, motoneurones and Renshaw cells. Co-axial electrodes were used to obtain intracellular records from motoneurones whilst simultaneously applying amino acid to the external surface of these cells. All substances were prepared for use in the electrode as near-saturated solutions in dilute NaOH or HCl, so that the final pH was appropriate for their iontophoretic application as either cations or anions. Table 1 lists the substances used, the approximate molarity, pH and nature of the solution and the predominant

Table 1

	Approx. molarity	pH	Solvent	Predominant ion species
L-Aspartic acid	2	8	NaOH	Anion
L-Glutamic acid	2	8	NaOH	Anion
Cysteic acid	1·5	8	NaOH	Anion
β-Alanine	4	2·5–3	HCl	Cation
γ-Amino-*n*-butyric acid	4	2·5–3	HCl	Cation
Taurine	0·7	8	NaOH	Anion
Acetylcholine bromide	2	4	Water	Cation
Dihydro-β-erythroidine hydrobromide	0·5	4	Water	Cation

ion species present in each case. It will be convenient, as in the earlier paper (Curtis *et al.* 1959*b*), to refer to currents as 'cationic' or 'anionic' when active cation or anion respectively is passed from the electrode.

RESULTS

As in the earlier report (Curtis *et al.* 1959*b*) concerning the depressant amino acids, all results are qualitative but the impression was gained that there was little quantitative difference between glutamic, aspartic and cysteic acids. These substances have been applied in their anionic form to interneurones, motoneurones and Renshaw cells.

Interneurones

Records have been obtained from over sixty individual interneurones located in the dorsal horn or intermediate nucleus of the spinal cord and activated synaptically by volleys in afferent fibres. All such cells have been fired by glutamate, aspartate or cysteate ions; in many cases all substances have been applied to the same cells with similar results. Figure 1 illustrates spike potentials from such an interneurone, recorded by means of the central barrel of a five-barrelled electrode. This cell was fired orthodromically by impulses in the high-threshold fibres of the sural nerve and typically the orthodromic spikes (Fig. 1*A–F*, *O*) were repetitive and superimposed upon a negative focal potential produced by the simultaneous activation of many neighbouring cells. The details of these spikes are shown more clearly in Fig. 1*G*, the diphasic spike being initially negative and followed by a smaller positive potential. This form of spike potential (cf. Brooks & Eccles, 1947) indicates that the soma of the cell, being a 'sink' for current flowing externally during its active spike phase, becomes a 'source' as the spike propagates along the axon.

The lower line in Fig. 1 records the current passing through a barrel of the electrode containing sodium aspartate. Initially a cationic current of 20 nA prevented the aspartate anion from diffusing out of the electrode, but in Fig. 1*B*, ▲ this current was reversed and gradually increased to a maximum of 240 nA (Fig. 1*C*, ▲) in 3·8 sec, thus passing an increasing amount of aspartate anion into the extracellular space. The current was then progressively decreased and terminated in Fig. 1*D*, ▲ 3 sec later. This cell was firing spontaneously at a low frequency (Fig. 1*A*), but during the application of the aspartate ion the rate of firing was increased to a maximum of 72/sec and then decreased to the initial frequency (Fig. 1*E–F*), the rate following closely the magnitude of the current used to pass aspartate from the electrode. Further, as the rate of firing increased, the spike potential was changed in shape, the positive phase increasing in size and the preceding negativity decreasing. These effects were readily reversible, the spikes of Fig. 1*E* showing a progressive decrease in

the positive phase; those of Fig. 1*F* were identical with the spikes of Fig. 1*A*.

Similar observations are shown in Fig. 2, this cell being fired repetitively in response to a single orthodromic volley in the peroneal nerve (Fig. 2*A*, *F*, *G* and *L*). Although the spike shape is not obvious in Fig. 2, the en-

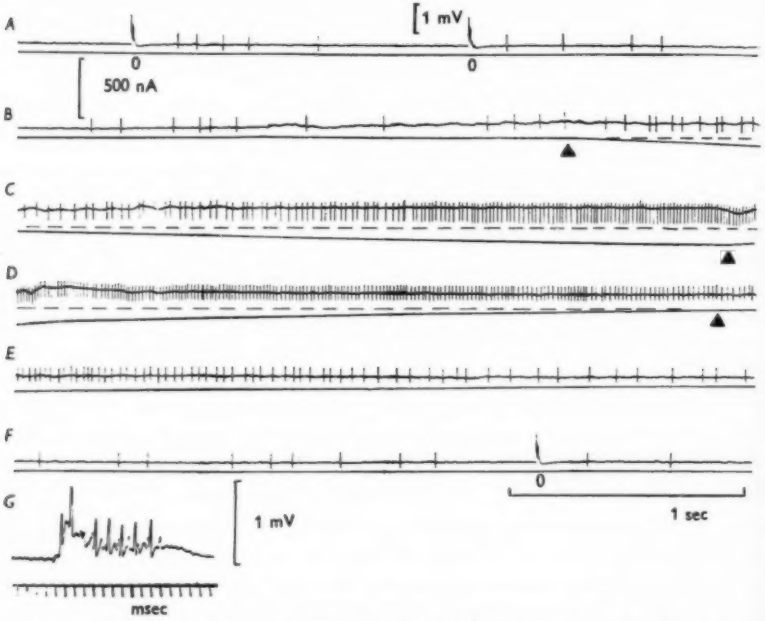

Fig. 1. Upper beam, potentials recorded extracellularly near a single spinal interneurone located in the dorsal horn of the seventh lumbar segment and fired orthodromically (O) by impulses in the sural nerve. The series are continuous from *A* to *F* and were recorded on film moving parallel to the X axis of the oscilloscope. The orthodromic response is shown in *G* at faster sweep speed. These responses were recorded by means of the central barrel of a five-barrelled electrode, one other barrel of which contained sodium aspartate.

Lower beam indicates the anionic current passing aspartate from the electrode. The current commenced at *B*▲, reached a maximum of 240 nA at *C* and thereafter was reducing, ceasing at *D*▲. Voltage calibrations: 1 mV for upper beam *A–F*; 500 nA for lower beam; 1 mV for *G*. Time marker, 1 sec for *A–F*; msec for *G*.

larged record of Fig. 3*A*, which corresponds to that of Fig. 2*G*, shows that the orthodromic spikes were diphasic. Each of the two series *A–F* and *G–L* was recorded on moving film, and the top of one column is continuous with the bottom of the next, there being less than 5 msec between sweeps. At the marker in Fig. 2*B* (▲) glutamate ion was passed, as an anion, into the environment of the cell with a current of 53 nA; this current

being maintained at a constant level until it was terminated in Fig. 2*E* (▲). After a latency of about 600 msec the cell commenced to fire (Fig. 2*B*), the frequency gradually increasing to a maximum of about 75/sec. This frequency was maintained fairly uniformly until the cessation of the iontophoretic current (Fig. 2*E*, ▲). The cell then ceased firing in less than 250 msec. During the firing evoked by glutamate the orthodromic stimulus produced only one or two spikes; these, however, were followed

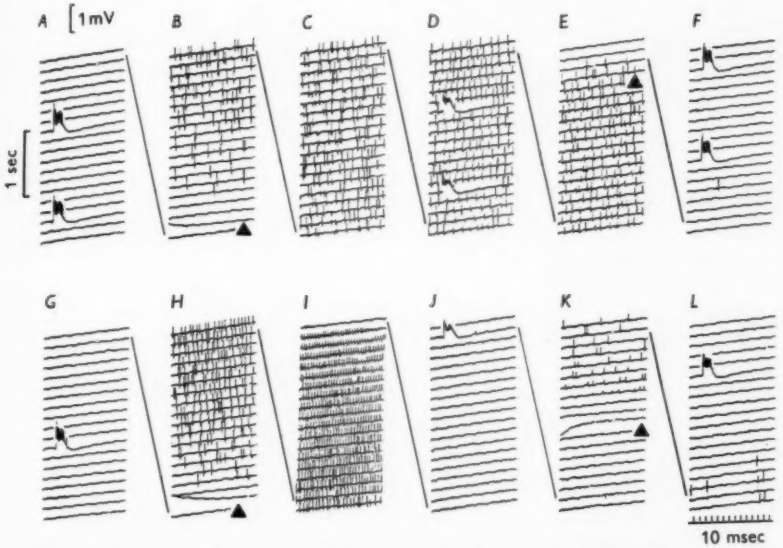

Fig. 2. Extracellular responses of an interneurone located in the seventh lumbar dorsal horn and fired synaptically by a volley in the peroneal nerve. The responses were recorded on film moving parallel to the Y axis of the oscilloscope, there being less than 5 msec between sweeps. The rate of film movement is indicated by the vertical time bar to the left of *A*. The responses within the two series *A–F* and *G–L* are continuous but commence at the bottom of each column so that the top of a column is continuous with the bottom of the next in line. The records were made by means of one barrel of a double electrode, the other barrel containing sodium glutamate. Anion was prevented from diffusing out of the electrode by means of a cationic current of 30 nA. In the series *A–F* an anionic current of 53 nA was passed for a time indicated by the two signs (▲); similarly in the series *G–L* a current of 120 nA was used. Voltage calibration, 1 mV.

by a short period of quiescence lasting 60–90 msec (Fig. 2*D*). This 'pause' is similar to that observed when Renshaw cells were fired synaptically whilst being activated by iontophoretically applied acetylcholine (Curtis & R. M. Eccles, 1958*a*) and has also been observed following the orthodromic excitation of many interneurones when these cells have a high

'back-ground' or spontaneous rate of discharge. After the cessation of the application of glutamate the orthodromic responses of the cell (Fig. 2*F*) were identical with those of Fig. 2*A*.

In the series Fig. 2*A–F* the chemically evoked spikes were very similar to those produced synaptically and remained constant in shape throughout the series. The size of the positive phase of the spike potential was, however, a little larger than that of the synaptic spike. When a larger

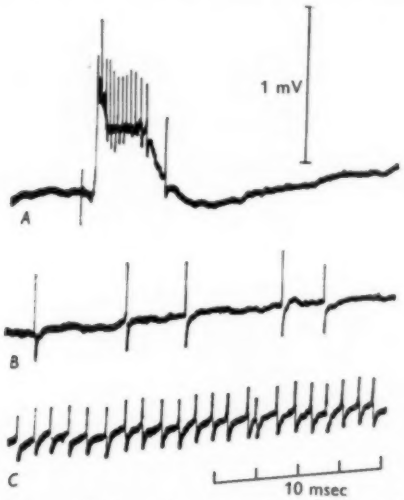

Fig. 3. Extracellular records from the same cell as illustrated in Fig. 2 but at higher magnification. *A*, orthodromic responses of Fig. 2*G–L*; the spikes have been retouched because of partial obliteration in the original records by the succeeding sweeps. *B, C*, responses evoked by glutamate ion; *B* corresponding to the top trace of Fig. 2*H* and *C* to portion of the fourth trace from the top of Fig. 2*I*.

current of 120 nA was used to pass glutamate from the electrode (Fig. 2*G–L*) this same cell fired at an increasing frequency (Fig. 2*H, I*), the negative phase of the spike becoming smaller and eventually the spike becoming replaced by a small positivity (Fig. 2*I*). Details of the spikes are shown in Fig. 3, Fig. 3*B* corresponding to portion of the upper traces of Fig. 2*H* and Fig. 3*C* to portion of the fourth trace from the top of Fig. 2*I*.

The maximum frequency of this cell was 220/sec (to be compared with the initial frequency following synaptic activation of 900/sec), and after the conversion of the spike to a small positive potential no further responses were evoked either by the continuing application of glutamate or by an orthodromic stimulus (Fig. 2*J*). Following the termination of the iontophoretic current (Fig. 2*K*, ▲) small negative spikes appeared within 500 msec. These increased in size, becoming not unlike the spikes produced

earlier either by glutamate (Fig. 2*H*) or by orthodromic activation. When tested 3·5 sec later the orthodromic response was normal (Fig. 2*L*).

Thus the two cells illustrated in Figs. 1–3, and indeed all those interneurones observed in similar circumstances, were fired repetitively after a latency of 0·25–0·5 sec by either glutamate, aspartate or cysteate ions applied iontophoretically. Most cells observed were fired at low frequency when the 'backing potential' was removed from the electrode. This establishes beyond reasonable doubt that these acids have an excitant effect apart from any possible action of the flow of current. Further responses of the cells depended upon the current used and thus upon the amount of anion applied. When the iontophoretic current was low and hence also the maximum frequency, as in Figs. 1 and 2*A–F*, the cell continued firing during the application of the amino-acid anions and ceased within 0·25–0·5 sec of the termination of the current. The evoked spikes were often very similar to those produced orthodromically, namely, a negative–positive diphasic spike, the negativity predominating. This type of response is very similar to that recorded from Renshaw cells activated by iontophoretically applied acetylcholine (cf. Curtis & R. M. Eccles, 1958*a*). In some cases, however, as in Fig. 1, the negative component of the spike was decreased in size and the positivity correspondingly increased. In those cells where firing was maintained by a continuous application of the active anion, the orthodromic responses were partially blocked, there being fewer spikes than usual.

When larger amounts of the anions were passed, the cells were fired at higher frequency and the spikes were rapidly altered in one of two main ways. Either there was a simultaneous progressive reduction in both negative and positive phases or else the negative phase was progressively reduced in size in relation to the positivity. The final outcome, however, was a small positive spike which itself eventually disappeared. Simultaneously, the orthodromic responses of these cells were blocked. When this stage was reached, no response could be elicited as long as the iontophoretic current was maintained. Shortly after its cessation, however, a few spikes were observed having a nearly normal ratio between negative and positive spike size. At this time also normal orthodromic responses could be evoked. These alterations in spike configuration were not due to the flow of current from the electrode, since even greater currents could be passed through the NaCl-containing barrel of the electrode without effect. Although on occasions the responses of cells could be modified by current alone, these effects began and ended simultaneously with the commencement and cessation of the current and were thus easily differentiated from the chemically modified activity seen in Figs. 1–3. When multibarrel electrodes were used, it was observed that if current

flow through one barrel influenced the behaviour of cells in this fashion, current flowing in the same direction in other barrels reproduced the effect, independently of the solutions contained therein.

The seemingly dual excitant-depressant actions of these amino-acid ions were also shown by their effects upon the field potentials recorded in the vicinity of groups of cells activated synaptically. When recorded extracellularly in the dorsal horn, the orthodromic excitation of groups of cells is signalled by comparatively large and slow negative potentials upon which are superimposed the faster spike potentials of single cells

Fig. 4. Focal potentials recorded within the seventh lumbar segment by means of double-barrel electrodes. *A–D* recorded in the dorsal horn and evoked by maximal stimulation of the peroneal nerve. After the control record *A*, a current of 100 nA passed glutamate ion from the electrode. *B* and *C* were recorded 12 and 19 sec later; the anionic current was then terminated and the record *D* taken 12 sec later. *E–H* from another experiment, recorded within the ventral horn in response to maximal stimulation of the segmental ventral root. After the control record *E*, a current of 100 nA passed aspartate ion from the electrode. *F* and *G* were recorded 18 and 29 sec later; the current was then terminated and the record *H* taken 20 sec later.

located close to the tip of the recording electrode. These focal potentials were effectively reduced in magnitude by both β-alanine and γ-amino-*n*-butyric acid (cf. Curtis *et al.* 1959*b*). Similarly when either glutamate, aspartate or cysteate ions were applied the focal potentials were also reduced in size. Typically the onset of the effect was slower than that observed using GABA, and occasionally at an early stage there was a slight increase in the recorded potential, presumably due to the activation of individual cells. Following the cessation of the anionic current applying the amino-acid ions the field potentials rapidly regained their original size, the rate of recovery being very similar to that observed after the cessation of the cationic current applying GABA. As with GABA and β-alanine (cf. Curtis *et al.* 1959*b*), it was possible to convert negative

field potentials into positive potentials of similar time course. The negative focal potential of Fig. 4*A* was produced by strong stimulation of the peroneal nerve. Twelve seconds after the onset of the passage of glutamate from the electrode this potential was virtually abolished (Fig. 4*B*) and a further 7 sec later it was converted into a positive potential (Fig. 4*C*). Following the cessation of the anionic current, the field potential recovered completely (Fig. 4*D*). Curtis *et al.* (1959*b*) have reported that some of the neutral amino acids, for example β-alanine, will suppress the spikes evoked in spinal neurones by orthodromic volleys or by glutamate ion. Since these experiments were necessarily performed with multibarrel electrodes, and because in most cases the excitant acids were applied as anions and the depressant acids as cations, such interference in the action of the excitants could conceivably be merely a consequence of the opposite current used to apply the supposed depressant. This possibility was discounted by applying both acids as anions. Thus taurine applied as an anion from alkaline solutions effectively prevented the excitation of interneurones by any one of the excitant amino-acid ions. Technical difficulties precluded the application of β-alanine and GABA as anions from alkaline solutions in multibarrel electrodes, although when passed from freshly prepared alkaline solutions through co-axial electrodes these substances were effective depressants of neuronal activity (Curtis & Watkins, 1960).

Motoneurones

When glutamate, aspartate or cysteate ions were applied from one barrel of a multibarrel electrode into the vicinity of motoneurones fired either orthodromically or antidromically, the evoked field potentials, recorded by means of another barrel of the electrode, were reduced in size in a fashion similar to that observed with the field potentials generated by the firing of interneurones. The negative potential of Fig. 4*E* was recorded from the ventral horn of the L7 segment of the spinal cord in response to maximal ventral root stimulation. The recording was made by means of one barrel of a double electrode; the other barrel contained the sodium salt of L-aspartic acid, the anion being prevented from diffusing out of the electrode by a backing current of 50 nA. When this cationic current was reversed and increased to 100 nA, thereby passing the aspartate ions from the electrode, the field potential was decreased in size, being abolished in 12 sec (Fig. 4*F*) and then converted into a positive potential (Fig. 4*G*). Several seconds later this anionic current was terminated and the field potential recovered fully in 20 sec (Fig. 4*H*).

When co-axial electrodes were used, allowing simultaneous intracellular recording whilst applying glutamate, aspartate or cysteate ions externally to the surface of the membrane of the impaled cell, a membrane depolariza-

tion was recorded. The responses of Fig. 5 are from a gastrocnemius cell and were recorded with an electrode containing 0·6 M-K_2SO_4 solution, of tip diameter less than 0·5μ. This inner shaft of a co-axial assembly projected 60μ beyond the 8μ diameter outer electrode which contained the sodium salt of L-aspartic acid. The cell had a resting potential of 66–72 mV

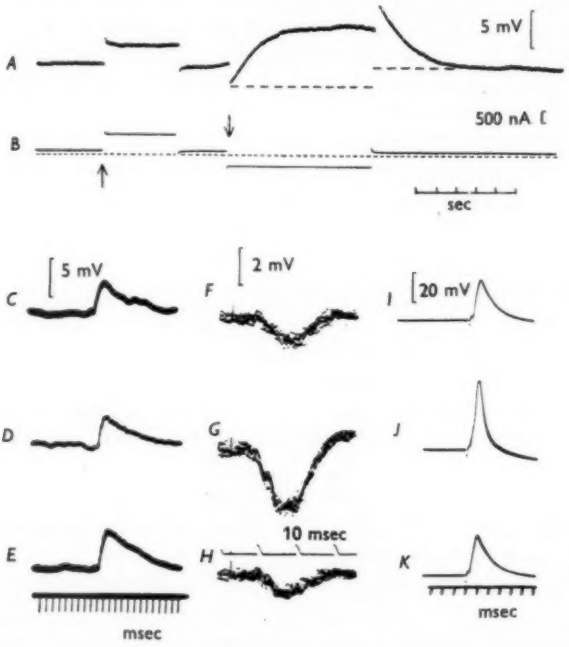

Fig. 5. *A*, the alteration in the resting potential of a gastrocnemius motoneurone recorded by means of an electrode, containing 0·6 M–K_2SO_4, which was the inner projecting barrel of a co-axial assembly; the resting potential of the cell was 68 mV over the time recorded; upward deviation indicates depolarization. *B*, recorded simultaneously with *A*, indicates the current flowing in the outer barrel of this electrode, which contained sodium aspartate solution. The dotted line indicates zero current, there being originally a cationic current of 200 nA flowing. This was increased by 720 nA (upward arrow) and later reversed so that a total anionic current of 520 nA passed aspartate from the electrode (downward arrow). *C–K*, intracellular responses recorded from this cell; *C*, *F* and *I*, control monosynaptic EPSP, polysynaptic IPSP and antidromic spike generated by gastrocnemius nerve, peroneal nerve and ventral root stimulation, respectively; *D*, *G* and *J* evoked by the same respective stimuli but during the depolarization produced by anionic currents of 500, 520 and 510 nA, respectively; *E*, *H* and *K*, control responses after the recovery of the membrane potential. Voltage calibration: 5 mV for *A* (above A); 5 mV for *C–E* (above C); 2 mV for *F–H* (above F); 20 mV for *I–K* (above K). Current calibration, 500 nA for *B*. Time marker, 1 sec for *A*, *B*, msec for *C–E* and *I–K*, 10 msec for *F–H*.

which remained fairly constant for a period of 2 hr. Figure 5*A*, recorded with a DC amplifier, shows the alterations in this resting potential (intracellular depolarization recorded upwards) produced by the passage of current through the outer barrel of the electrode, the corresponding alterations in current being shown in Fig. 5*B*, where anionic currents are indicated by a downward deflexion. A cationic current of 200 nA was flowing through the outer barrel throughout in order to prevent the free diffusion of the aspartate anion therefrom. Initially (upward arrow Fig. 5*B*) an additional cationic current of 720 nA was passed and the resultant apparent alteration in membrane potential in a depolarizing direction, recorded by the inner electrode, was a consequence of current flow in the resistance of approximately 4 kΩ between the tips of the two electrodes. A similar potential was recorded when both electrodes were extracellular, anionic and cationic currents producing mirror-image potentials of identical size. In Fig. 5*B* (downward arrow), an anionic current of 520 nA was then passed and the broken line of Fig. 5*A* plots the alteration in potential which would have been expected from this current flow alone. However, the recorded potential diverged from that predicted by at least 7·5 mV in the direction of a membrane depolarization. When the flow of current was terminated, the resting potential returned to its original value with a time course very similar to that with which the depolarization arose. Similar depolarizations were obtained with smaller and larger currents. Cessation of the 'backing' current itself resulted in a membrane depolarization of about 1·5 mV, which was probably due to the uncontrolled diffusion of aspartate ion from the electrode.

Results similar to those illustrated were obtained with all motoneurones tested with these amino acids, a total of 12 cells being so investigated. Thus glutamate, aspartate and cysteate ions depolarize the membrane of motoneurones, the time course and size of this action being dependent upon the current used and the distance between the point of application and the cell membrane. Depolarizations were maintained unchanged for 10–30 sec and currents as large as 1 μA were used.

In addition to the alterations in membrane potential level produced by these ions, the effects upon post-synaptic responses were determined. During depolarizations such as are illustrated in Fig. 5*A*, excitatory post-synaptic potentials (EPSP) evoked by stimulation of group Ia afferent fibres were slightly reduced in size (Fig. 5*C–E*), whereas inhibitory post-synaptic potentials (IPSP—Fig. 5*F–H*) were increased. It has been demonstrated that for motoneurones the equilibrium potential for the IPSP is close to the resting potential (cf. Coombs, Eccles & Fatt, 1955*b*), whereas that of the EPSP is near zero potential (Coombs, Eccles & Fatt, 1955*c*). Consequently a depolarization of even 10 mV would be expected

to decrease the EPSP very little, but should greatly increase the IPSP. If, however, this depolarization was associated with an increased membrane permeability to certain ions, the resultant increase in membrane conductance might be expected to reduce the size of both the EPSP and IPSP. Thus the effects observed in Fig. 5*C–H* do not necessarily indicate a specific alteration in the process of synaptic inhibition, but are consistent with the occurrence of a membrane depolarization, perhaps modified by an associated conductance change.

The depolarization of motoneurones produced in this way was never sufficient to fire the cell, presumably because of the difficulty of obtaining a sufficiently high concentration of these acids over a large enough area of

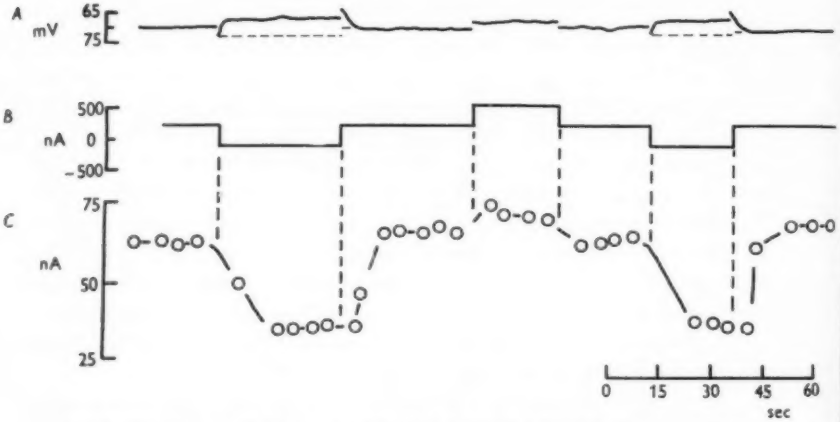

Fig. 6. Recorded from the same cell as Fig. 5, *A* and *B* again indicating membrane potential and iontophoretic current, respectively. The broken line of *A* indicates the membrane potential alteration which would result from the flow of current alone (see text). *C*, magnitude of a current pulse of 0·5 msec duration which was just threshold for directly exciting the cell when applied via the intracellular electrode; these tests were made simultaneously with the responses of *A* and *B*, the vertical broken lines indicating the beginning and end of the current pulses. Voltage calibration, mV, indicating resting potential, depolarization again being an upward deviation; current calibration, *B*, nA, anionic current being a downward deviation; the backing current of 200 nA is indicated by the difference between the current record and the zero current: *C*, nA.

membrane. However, the depolarization could be summed with either a subthreshold EPSP or an antidromic IS spike to produce a full soma-dendritic spike. For the cell illustrated in Fig. 5, stimulation of the axon in the segmental ventral root resulted in an intracellular spike response of 27 mV (Fig. 5*I*) which had the characteristics of an IS spike (Coombs, Curtis & Eccles, 1957*a*), but was probably considerably attenuated by the

comparatively large input impedance of the recording system (cf. Curtis *et al.* 1959*b*). During the application of aspartate, the identical ventral root stimulus produced a full SD spike (Fig. 5*J*), again attenuated. This result was reversible, an IS spike being again produced following the cessation of the iontophoretic current (Fig. 5*K*).

The direct excitability of impaled cells could be tested by means of rectangular current pulses passed through the inner electrode (cf. Coombs, Curtis & Eccles, 1959). In Fig. 6*C* are plotted results from the same cell as Fig. 5, each point indicating the magnitude of a current pulse of 0·5 msec duration which produced a direct spike potential in 50 % of 20 tests. As expected, during the depolarization produced by aspartate ion (alterations in membrane potential, Fig. 6*A*; outer electrode current, Fig. 6*B*) a smaller current pulse was necessary to evoke a spike than normally. There was only a slight alteration in the pulse when a 'depolarization' was recorded by the intracellular electrode as a cationic current flowed through the outer barrel. These results therefore demonstrate that aspartate, glutamate and cysteate ions applied iontophoretically to the surface of motoneurones each produce a depolarization which is associated with changes in post-synaptic potentials, and with an increase in the excitability of the cells.

Renshaw cells

Renshaw cells, located in the ventral horn of the spinal cord, are activated by acetylcholine released synaptically from the terminals of axon collaterals or applied iontophoretically from a nearby micro-electrode (Curtis & R. M. Eccles, 1958*a*, *b*). They are also very effectively fired by aspartate, glutamate and cysteate ions. For example, Fig. 7 illustrates responses recorded from a Renshaw cell which was fired repetitively by a single ventral root volley (Fig. 7*A*). These spike potentials were recorded from the central barrel of a five-barrel electrode. When acetylcholine was passed as a cation from one of the barrels of this electrode, the cell was fired repetitively (Fig. 7*B*) after a brief latency of 200 msec, continued to fire throughout the application and ceased within 250 msec after termination of the current (not shown). Similarly when glutamate was passed from another barrel (Fig. 7*C*) the Renshaw cell was again fired. It will be noted that during its action the effects of synaptic stimulation of this cell were slightly accentuated. When larger currents were used to pass either aspartate, glutamate or cysteate ions, Renshaw cells again were fired but rapidly ceased to respond both to synaptic and to chemical stimulation. Recovery was rapid after cessation of the amino-acid application. This type of response is identical to that found with interneurones and was illustrated in Fig. 2*H–J*. The finding that aspartate, glutamate and cysteate ions can also excite Renshaw cells has proved of great value in

determining the site of action of these amino-acid ions. It has already been demonstrated that the activation of Renshaw cells by acetylcholine is blocked both by dihydro-β-erythroidine (DHE) (cf. Curtis & R. M. Eccles, 1958*b*) and by the depressant monocarboxylic amino acids (Curtis *et al.* 1959*b*). However, although β-alanine and GABA prevent both aspartate and glutamate from firing Renshaw cells, DHE has no such effect. Thus, after the records of Fig. 7*A–C* DHE was passed from one barrel of the electrode, and 30 sec later *D*, *E* and *F* were recorded under the same circumstances as *A*, *B* and *C*, respectively. Although DHE depressed the firing of the cell produced by a ventral root volley (*D*) and almost completely abolished the activation of the cell by acetylcholine (*E*), the firing produced by glutamate was unaltered (*F*).

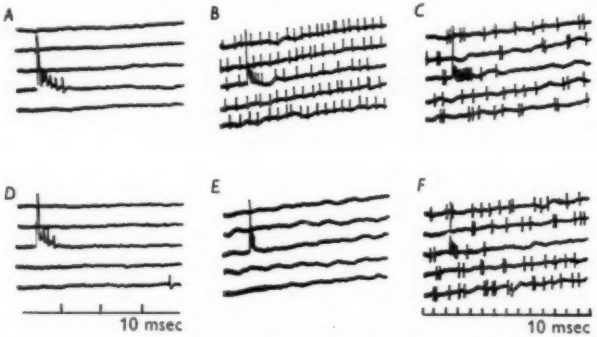

Fig. 7. Extracellular spike potentials of a Renshaw cell recorded by means of the central barrel of a five-barrel electrode and photographed on moving film in a similar fashion to Fig. 2. *A* and *D*, stimulation of the segmental ventral root; *B* and *E*, application of ACh with cationic current of 70 nA; *C* and *F*, application of glutamate ion with an anionic current of 110 nA: *A–C* before, *D–F* 30 sec after a current of 120 nA commenced to pass dihydro-β-erythroidine into the vicinity of the cell. (Spikes retouched.) Time marker, 10 msec for all records; for *A* and *D* below *D*, for *B*, *C*, *E* and *F* below *F*.

Enzyme studies

After the application of a chemical substance to the surface of a cell, either from an electrode or from presynaptic terminals, the fall in concentration of active material at any one point will depend not only upon 'free diffusion' (cf. Eccles & Jaeger, 1958) but also upon the rate of inactivation of the substance, be it enzymic or by a process of adsorption upon inert macromolecular surfaces. If the substance has an effect upon the postsynaptic membrane, the decay of this action will have a time course dependent upon both the rate of fall of the concentration of the substance and the stability of the receptor-substance complex.

Studies upon Renshaw cells (Curtis & R. M. Eccles, 1958*b*) have shown the importance of cholinesterase in the removal of acetylcholine released from synaptic endings or applied iontophoretically from a nearby electrode. The administration of an anticholinesterase either intravenously (Eccles, Eccles & Fatt, 1956) or locally from an electrode (Curtis & R. M. Eccles, 1958*b*) prolongs the action of synaptically released acetylcholine. Similarly the effect of iontophoretically applied acetylcholine is intensified and prolonged, this prolonged action being similar in duration to that observed normally for cholinergic drugs such as nicotine and carbachol, upon which cholinesterase has no action.

In the absence of anticholinesterase the action of iontophoretically applied acetylcholine upon a single Renshaw cell ceases within 200–500 msec (cf. Curtis & R. M. Eccles, 1958*a*, Figs. 2, 3, 5; 1958*b*, Fig. 9). Since the cessation of the responses of single interneurones and Renshaw cells produced by glutamate or aspartate ions (e.g. Fig. 2) also occurs within 500 msec of the termination of the applying current, there arises the possibility that these acids too are removed not only by free diffusion but also by enzymic destruction. On the other hand, the rapid termination of the effect could be a reflexion of the instability of the receptor-amino acid complex. This latter possibility is strengthened considerably by the similarity in the time course of cessation of activation of interneurones and Renshaw cells by L-glutamate, D-glutamate, L-aspartate, D-aspartate and L-cysteate ions, for it seems improbable that any one enzyme system could remove all these acids at such a similar rate.

Although it has been suggested that the process of diffusion is adequate to clear the subsynaptic regions following the release of excitatory substance upon motoneurones (Eccles & Jaeger, 1958), the observation that cholinesterase is concerned in the removal of acetylcholine from the synaptic sites upon Renshaw cells raises the possibility that similar processes are concerned with transmitter removal elsewhere in the nervous system. Consequently the identification of a substance as a true excitatory transmitter would be aided by a comparison of the responses of cells evoked both by orthodromic synaptic excitation and by iontophoretic application of the respective agent in both the presence and absence of appropriate enzyme inhibitors. The prolongation of both types of response by an inhibitor of an enzyme, for which the test substance is known to be a substrate, would suggest that the naturally occurring transmitter and the applied agent were identical, especially if the enzyme system had a high specificity towards the particular substance.

Various metabolic pathways involving glutamic and aspartic acids have been described (Waelsch, 1957). In view of the apparent dual action of the anions of these acids in exciting and then depressing neuronal activity, it is

TABLE 2

Substance	Solution	Enzyme	References
Sodium-*p*-chloromercuri-benzoate	H_2O	Glutamic dehydrogenase	Krebs, 1951
		Glutamic-pyruvic transaminase	Cohen, 1951
		Glutamic-oxaloacetic transaminase	Cohen, 1951
		Glutamine synthetase	Meister, 1956
		Monoamine oxidase	Zeller, 1951
		General D-amino-acid oxidases	Krebs, 1951
Isoniazid	H_2O	Glutamic-oxaloacetic transaminase	Sakai, 1954
(Isonicotinic acid hydrazide)		Monoamine oxidase	Zeller, Barsky, Fouts, Kirchheimer & Van Orden, 1952
		Diamine oxidase	Zeller, Barsky, Fouts, Kirchheimer & Van Orden, 1952
Iproniazid	H_2O	Glutamic-oxaloacetic transaminase	Sakai, 1954
(1-isonicotinoyl-2-iso-propylhydrazine phosphate)		Monoamine oxidase	Schayer, 1953
		Diamine oxidase	Zeller *et al.* 1952
Carbonyl group reagents (Phenylhydrazine HCl; hydroxylamine HCl; semicarbazide HCl; thiosemicarbazide HCl; thiocarbohydrazide HCl)	H_2O	Glutamic-pyruvic transaminase	Braunshtein, Azarkh & Mogilevskaya, 1955
		Glutamic decarboxylase	Killam, 1957; Killam & Bain, 1957
		Diamine oxidase	Zeller, 1951
Sodium benzoate	H_2O	Glutamic dehydrogenase	Takashina, 1956
		General D-amino-acid oxidases	Krebs, 1951
		Diamine oxidase	Zeller, 1951
Quinine HCl	HCl	Esterases including cholinesterase	Ammon & Jaarma, 1950; Augustinsson, 1950
		General D-amino-acid oxidases	Krebs, 1951
Ephedrine HCl	H_2O	Monoamine oxidase	Zeller, 1951
p-Phenylenediamine HCl	H_2O	Glutamic-oxaloacetic transaminase	Cohen, 1951
		Glutamic-pyruvic transaminase	Cohen, 1951
Sodium fluoride	H_2O	Glutamine synthetase	Cohen, 1952
		Esterases	Ammon & Jaarma, 1950
Sodium cyanide	H_2O	L-Amino acid decarboxylases (including glutamic decarboxylase)	Schales, 1951
		D- and L-amino acid oxidases	Krebs, 1951
		Glutamine synthetase	Meister, 1956
		Diamine oxidase	Zeller, 1951
DL-Methionine sulphoxide	HCl pH_3	Glutamine synthetase	Meister, 1956
DL-Methionine sulphoximine	HCl pH_3	Glutamine synthetase	Meister, 1956

natural to consider the enzymes responsible for α-decarboxylation in this respect, since the products of such enzymic action, γ-amino-*n*-butyric acid and β-alanine, respectively, have a powerful depressant action upon neurones (Curtis *et al.* 1959*b*). It might be considered that the eventual blocking action of the excitant amino-acid ions depended upon their conversion to the related depressant amino acids. It is also necessary, however, to take into account the other enzyme systems that might be involved. The various enzyme inhibitors used in attempting to assess whether or not these substances were removed from the site of application enzymically are listed in Table 2, together with the nature of the solution used, the enzymes which amongst others might thereby be inhibited, and the reference for this inhibition. The substances were applied iontophoretically for periods of up to 3 min and the effects determined both upon the orthodromic and glutamate-, aspartate- or cysteate-evoked spikes of interneurones. In particular observations were made upon the frequency of firing, the duration of response after the cessation of stimulation and the rate at which the applied ions either diminished field potentials or produced a depolarization type of block after initially exciting cells. Altogether records were obtained from over 20 cells, most being activated by glutamate ion. In no instance was any action of these enzyme inhibitors observed either on synaptically or on amino-acid-evoked spikes.

DISCUSSION

Extensive investigations have indicated that the production of the spike potential of a neurone by excitatory synaptic action involves two stages (Coombs, Curtis & Eccles, 1957*b*). Initially, as the result of transient localized increases in membrane permeability to all ions (Coombs, Eccles & Fatt, 1955*c*), an EPSP is set up which spreads electrotonically over the surface membrane of the cell. Secondly, when this depolarization reaches a threshold value, a spike potential is initiated at the region of the axon hillock and propagates along the axon and also into the body of the cell. The ionic mechanisms responsible for this spike are probably very similar to those producing a spike potential in a nerve fibre (cf. Hodgkin, 1958). The observations of the excitatory effects of glutamate, aspartate and cysteate ions upon spinal neurones can be interpreted on the basis of their active depolarization of the neuronal membrane. Not only has this depolarization of motoneurones been demonstrated, but it has also been possible to show that the depolarization is associated with a lowered threshold for excitation, as tested by a direct stimulus. The accompanying alterations in the excitatory and inhibitory post-synaptic potentials can be fully explained by this depolarization, and it is unlikely that there is any specific alteration in excitatory or inhibitory synaptic transmission.

Direct evidence has not been obtained from interneurones by means of intracellular electrodes, but it may be presumed that the primary cause of the discharge of impulses by these cells is also a membrane depolarization.

It is unlikely that the depolarizing action of the applied amino-acid ions is due to their passing into the intracellular phase, therein behaving as free ions or as sources of metabolic energy. Neuronal membrane is generally regarded as being impermeable to glutamate ion (Shanes, 1958). Moreover, considerable amounts of glutamate have been passed intracellularly into motoneurones (Coombs *et al.* 1955*a*) without producing much alteration in the resting potential. It is also unlikely that the activity of these substances is associated with the removal of calcium from the extracellular fluid around neurones, since many compounds which are more powerful calcium chelators have no excitant action upon spinal neurones when applied iontophoretically (Curtis, Perrin & Watkins, 1960).

It may be considered that the application of the ions results in the formation at the surface of the cell of an amino-acid–receptor complex, the stability of which must be low in view of the rapid decay of the effect. An investigation of the structure–function relationships of a large series of neutral and acidic amino acids has led to certain deductions concerning the requirements of such possible receptor sites (Curtis & Watkins, 1960). The postulated complex formation must result in an increase of membrane permeability, which may be either specifically to sodium ions or perhaps more generally to several of the ions involved in the maintenance of the membrane resting potential. It is convenient to refer to the sodium 'carrier' as that system responsible for the influx of sodium ions during the rising phase of the spike potential, without necessarily implying acceptance of any of the current hypotheses as to how this actually takes place (Hodgkin, 1958; Shanes, 1958; Mullins, 1959). If, under the influence of the excitant amino-acid ions, areas of the membrane became more permeable to sodium ions by localized activation of the sodium 'carrier', the resultant current flow would depolarize the cell. A depolarization would also result if the permeability change involved other ions as well as sodium; in this case the action of these amino acids would be similar to that of acetylcholine on the end-plate membrane of the neuromuscular junction (Fatt & Katz, 1951; del Castillo & Katz, 1954) and of the excitatory transmitter on motoneurones (Coombs *et al.* 1955*c*). Differentiation between these two mechanisms by which a membrane depolarization can arise will depend upon further analyses, particularly of the 'equilibrium' potential towards which the depolarization is directed (cf. del Castillo & Katz, 1954; Coombs *et al.* 1955*c*).

The observed effects of aspartic, glutamic and cysteic acids and their presence in the nervous tissue in association with enzymes responsible

for their synthesis and break-down (cf. Waelsch, 1957) raise the question whether these or related substances could be specific excitatory transmitter agents of interneurones and motoneurones (Paton, 1958). The site of action of these substances therefore becomes important, for if they were excitatory transmitters they would attach to the specific receptor sites located beneath excitatory synaptic endings. On the other hand, however, these acids might function as non-specific excitants by an action elsewhere upon the post-synaptic membrane. The observation that, although dihydro-β-erythroidine blocked the cholinergic excitation of Renshaw cells, the excitation by glutamate (Fig. 7), aspartate and cysteate was unaltered, leads to the conclusion that these ions do not combine with receptor sites specialized for combination with acetylcholine. This conclusion is supported by the finding that the amino-acid ions also depolarize interneurones and motoneurones, for these cells are non-cholinoceptive (Curtis, Phillis & Watkins, unpublished observations). However, it is not yet certain whether acetylcholine is the only excitatory transmitter acting upon Renshaw cells. If this were so, the firing of non-cholinoceptive and purely-cholinoceptive neurones by the amino-acid ions would show these substances were not specific excitatory transmitters. On the other hand, if other substances in addition to acetylcholine normally excite Renshaw cells, then these acids or related compounds may be specific excitatory transmitter agents of these spinal neurones. It is considered unlikely, however, that either glutamic, aspartic or cysteic acids are excitatory transmitters. When these amino-acid ions were applied to the one interneurone, the intervals preceding the cessation of cell responses after the termination of the iontophoretic currents were virtually identical in each case, as indeed were the intervals found for both dextro- and laevoforms of glutamate and aspartate ions (Curtis & Watkins, 1960). This suggests that enzymic removal of the applied acids were not a significant factor in determining the duration of these intervals, and since it is reasonable to consider that specific enzymes occur near excitatory synapses for the destruction of synaptically released transmitters, it follows that probably none of these amino acids plays a part as excitatory transmitter substance in the mammalian spinal cord.

Whatever the mechanism by which the acidic amino-acid ions produce a membrane depolarization, this change in potential must be accompanied by an increase in the membrane conductance. Both the degree of membrane depolarization and the proportion of the surface area of the cell involved in the conductance change would be determined by the distribution and concentration of the amino acid around the cell. Spike potentials would be initiated if and when the depolarization was sufficient to reach the threshold of the initial segment region. However, the propagation of

the spike into the soma and dendrites of the cell might be modified or blocked by the depolarization of these regions. The physical size of motoneurones together with the possible considerable distance (up to 50μ) of the electrode applying the excitant to the membrane would be factors precluding the attainment of sufficient depolarization to fire these particular cells. When interneurones and Renshaw cells were surrounded by a substance capable of producing a membrane depolarization, the action could be detected only if and when this depolarization exceeded the spike threshold, for the method of extracellular recording permitted detection only of spike potentials. When spikes are generated, these cells would respond repetitively with a frequency determined both by the concentration of the active agent and by the duration of the recovery processes following the spike. Both interneurones and Renshaw cells respond repetitively to synaptic stimulation with frequencies as high as 1000–1200/sec, but these cells were rarely driven at frequencies beyond 600/sec either by the acidic amino acids or, in the case of Renshaw cells, by acetylcholine (Curtis & R. M. Eccles, 1958*a*). This suggests that the normal duration of the recovery process following spikes is not a factor limiting the rate at which these cells can be fired by iontophoretically applied agents, but it is possible that the recovery processes themselves are modified by the agents, thereby limiting the attainable frequencies.

When large currents were used to apply these amino-acid ions there was eventually an alteration in the shape of both the chemically and synaptically evoked spikes. The observation that the negative-positive extracellular spikes were either reduced in size or rendered predominantly positive before being abolished presumably indicates that areas of the cellular membrane became incapable of producing spike potentials. When a spike potential propagates over a cell membrane, a nearby electrode records a negative potential which is generated by current flowing in the extracellular medium from the inactive towards the active membrane (cf. Brooks & Eccles, 1947). If the spike also propagates down the axon of the cell this negativity is followed by a positive potential. Consequently, the area of the membrane observed by the electrode is initially a 'sink' for current and then a 'source'. This negative-positive potential sequence was almost invariably observed when interneurones were fired synaptically (Figs. 1*G*, 3*A*) and at low frequencies by the excitant amino-acid ions (Figs. 1, 2). However the electrophoretic application of the excitant amino-acid ions will cause a selective depolarization of the nearby regions of the neuronal membrane and consequently a partial or complete inactivation of those processes responsible for the initial phases of the spike potential (cf. Hodgkin, 1958). Hence the negative phase of the spike potential recorded extracellularly by another barrel of the multibarrel electrode assembly

would be depressed, and, if the depolarization was of sufficient intensity, the spike in this region would be abolished. In this instance the extracellularly recorded spike potential will exhibit only the later positive component, as in Fig. 2*I*. The local depolarizing action of the applied amino-acid ions would also depress excitatory synaptic potentials and even reverse the direction of the focal synaptic potential (negative to positive) recorded in close proximity to the site of application. If the whole of the cell membrane was sufficiently depolarized by the applied ions, a condition would be reached in which the inactivation was extensive enough to prevent the generation of spikes, both by the applied ions and by synaptic stimulation (cf. Fig. 2*J*).

The depressant action of the excitant amino-acid ions on spike potentials and synaptic potentials accounts satisfactorily for the comparatively slow abolition and even reversal of negative field potentials generated by the activity of groups of cells. This type of response was also observed when the depressant amino acids were applied under similar cicumstances (Curtis *et al.* 1959*b*), but in those instances the effect was more rapid. It is probable that the depressant action of acids such as β-alanine and GABA occurs because these substances render neuronal membranes more permeable to certain ions without producing an alteration in membrane potential (Curtis *et al.* 1959*b*). The action of the excitant amino-acid ions, on the other hand, is associated with a depolarization which leads initially to the production of spike potentials, but when observing the activity of groups of cells the excitation was but rarely seen, presumably because the asynchronous spike potentials of many cells tend to cancel each other. In addition to this excitatory action, the acidic amino-acid ions render the affected areas of neuronal membrane incapable of supporting a spike potential. When either type of amino acid is applied from an electrode into a whole population of neurones, two factors may be expected to influence the field potential evoked by synaptic or antidromic activation. First, neuronal membrane adjacent to the applying and recording electrodes may become inactive whilst other areas on the same cells remain capable of supporting a spike. Secondly, an increasing number of cells will become totally inactivated. Both these factors would reduce extracellularly recorded negative potentials produced by the flow of current through the extracellular medium. In addition, the former factor will involve the conversion of areas of membrane from sinks to sources of current, flowing to activated membrane elsewhere on the cells, and would thus account for the actual reversal of field potentials from negative to positive.

Another explanation of the eventual depressant action of these excitant amino-acid ions can be based upon the replacement of their depolarizing effect by another action either

previously masked or having a slower rate of onset. It may be envisaged, for example, that the membrane becomes inexcitable to both synaptic and chemical excitation by a process of desensitization (cf. Katz & Thesleff, 1957; Axelsson & Thesleff, 1958). Alternatively, as the corresponding neutral amino acids, GABA, β-alanine and taurine, depress spinal neurones and are capable of preventing the excitatory action of glutamate, aspartate and cysteate ions, it is possible that the post-synaptic membrane could become unresponsive to the excitants owing to the enzymic α-decarboxylation of these latter substances to the respective depressant amino acids. This, however, is unlikely for several reasons. First, the enzymic decarboxylation of all three acids and of the D- and L-forms of each, since these are equally active (Curtis & Watkins, 1960), would of necessity have to proceed at the same rate, which seems improbable. Secondly, iontophoretically applied enzyme inhibitors with actions upon glutamic decarboxylase had no effect upon the rate with which depression followed excitation. Finally, the observation that continued application of the excitant amino-acid ions to motoneurones produces a depolarization, which can be maintained, not only suggests that conversion of the excitant to a depressant (which does not depolarize) does not occur, but also renders extremely unlikely the desensitization of the neuronal membrane to the applied agent. It must be assumed that the maintained depolarization shown with motoneurones occurs also with interneurones and Renshaw cells and that the alterations in spike shape, together with the eventual abolition of spike response, is indicative of a progressive diminution of the area of neuronal membrane producing a spike.

The rapid recovery of interneurones and Renshaw cells after the application of excitant amino-acid ions is most probably due not only to the diffusional removal of these ions but also to the relative instability of the receptor–drug complex which results in depolarization. The observation that the recovery time after the depolarizing action of a variety of substances, including D- and L-aspartate, D- and L-glutamate, L-cysteate, β-aminoglutarate and β-hydroxy-DL-glutamate (Curtis & Watkins, 1960), is very similar to that occurring after the depressant action of related monocarboxylic, or sulphonic amino acids, renders unlikely their removal by enzymic means. Further, similar recovery times follow the excitant action of calcium chelating agents (cf. Curtis *et al.* 1960). Studies with enzyme inhibitors failed to detect any action upon either amino-acid or synaptically evoked discharges of interneurones. Indeed the failure of a range of inhibitors to potentiate the synaptic responses of these cells may indicate that the released transmitter is not one of these excitant amino acids. In this respect, however, several factors must be taken into consideration. In the first place the enzyme responsible for destruction of the released transmitter may not be sensitive to the inhibitors chosen, these having been selected because of their actions upon the known metabolic pathways of the amino acids. Secondly, synaptic transmitter agents may be released within diffusional barriers (cf. Eccles *et al.* 1956; Curtis & R. M. Eccles, 1958*b*) which can exclude iontophoretically applied enzyme inhibitors. This has been observed at Renshaw cells where decamethonium (C_{10}), although an effective anticholinesterase with respect to iontophoretically applied acetylcholine, has no such action upon the acetylcholine

released synaptically (Curtis & R. M. Eccles, 1958*b*). Finally, it is important to realize that the relative potency of these inhibitors is low when the activity is compared with that of an anticholinesterase such as prostigmine. This latter agent, which is very effective when applied iontophoretically near Renshaw cells, is a powerful inhibitor of cholinesterase in concentrations of 10^{-6}–10^{-7}M (Augustinsson, 1950). In contrast, most of the inhibitors used in this present investigation require concentrations of 10^{-3}–10^{-4}M (see refs. Table 2). It is possible to calculate that under steady-state conditions of iontophoretic application, with currents of up to 500 nA, concentrations such as would be necessary are probably obtained (Curtis *et al.* 1960), but the possibility exists that the absence of observable action of these enzyme inhibitors might indeed be due to a failure to reach a satisfactory concentration.

It remains to correlate these observations of the action of glutamate, aspartate and cysteate ions with the reports of other investigators. The higher concentration of glutamic acid in nervous tissue lends importance to this substance, but does not necessarily indicate that its function as a transmitter agent is more likely than that of the other acids. Hayashi (1952) was able to produce convulsions in dogs by injecting sodium glutamate into the grey matter of the cerebral cortex, and these effects were probably due to a direct depolarizing action upon neurones. The observations of Purpura, Girado, Smith, Callan & Grundfest (1959) concerning the actions of aspartic and glutamic acids upon the extracellularly recorded potentials in the cerebral and cerebellar cortex are also in accordance with the present results, as illustrated in Fig. 4*A–D*. Similar considerations apply to the observations of Van Harreveld (1959) concerning the initiation of spreading depression in the rabbit cortex by L-glutamic and some related dicarboxylic amino acids. Because the concentrations of the substances producing this effect were less than the over-all concentration of glutamic acid in the tissue, it was concluded that these compounds were effective by reason of their presence in the extracellular fluid around neurones and that the endogenous acids must be mostly intracellular. The present report of the action of glutamate ion on spinal neurones lends support to the postulate that spreading depression could result from the action of this acid upon cortical neurones. Further, Van Harreveld demonstrated that glutamic acid activated crustacean muscle, although the experimental method did not discriminate between an action at the neuromuscular junction and one on the muscle fibre directly.

The iontophoretic method of application used in these present experiments does not permit an accurate determination of the local concentrations which are obtained (cf. del Castillo & Katz, 1955). However, by assuming that this acid has a diffusion coefficient of a similar magnitude

to that of acetylcholine, it can be calculated that when currents of 100 nA are used, it is improbable that the steady-state concentration is as high as 10^{-2}M, apart from that in the region within 10μ of the tip of the electrode (cf. Curtis *et al.* 1960). The distance from the electrode tip to the cell upon which the action of the acid is being determined is always unknown, but it was often possible to move the electrode by as much as 50μ without altering appreciably the response of a single cell to the applied amino acid. Moreover, in the absence of iontophoretic currents cells responded to glutamate ion which merely diffused from the electrode. It has recently been demonstrated that neurones of the isolated, bisected spinal cord of the frog were excited by any of the excitant amino acids at concentrations of 10^{-4}–10^{-3}M, when these substances were added to the solution bathing the tissue (J. Kiraly & J. W. Phillis, unpublished observations). It can be concluded therefore that the spinal interneurones of the cat were fired by extracellular concentrations of glutamate ion lower than 10^{-2}M. Since the over-all concentration of free glutamic acid in the central nervous system of the cat is approximately 10^{-2}M (Strecker, 1957), this conclusion renders extremely improbable the uniform distribution of this acid within the intra- and extracellular spaces. The maintenance of the resting potential of neurones, dependent upon a differential distribution of ions, necessitates a large amount of intracellular organic anion (Hodgkin, 1958). For this reason, as well as the obvious one related to their actions when applied extracellularly, it is probable that a major proportion of these amino acids, the anions of which excite cells, is located intracellularly.

The demonstration that several acidic amino acids, naturally occurring in brain, can stimulate cells leads to a discussion of convulsive states, particularly as one of the biochemical disorders associated with epileptogenic foci of cortical tissue has been shown to be concerned with glutamic acid. Tower (1955, 1956) has discussed in detail the observed difference in the metabolic reactions of normal cortical slices and those of epileptogenic cerebral foci. It is at present impossible to determine whether any of these differences cause or are merely a result of the seizure state. It may be pointed out, however, that a defect in the system maintaining low extracellular concentrations of these substances could well be the direct cause of this aberrant excitation of cells. The observed diminution in the total glutamic acid of the slice cannot be taken as an objection to such an hypothesis, since this would be accounted for by the increased consumption of glutamic acid as a substrate during the disordered activity. Further, it has been suggested that the convulsive activity of certain hydrazides and of deficiency of vitamin B_6 is linked with the depression of brain glutamic decarboxylase activity (Jenney & Pfeiffer, 1958; Killam, 1957; Killam & Bain, 1957). The associated fall in the level of brain gamma-

amino-*n*-butyric acid has led to the suggestion that this substance has a function in regulating the activity of neural tissue, although Elliott (1958) has pointed out that the pyridoxine-dependent co-enzymes which are inhibited by hydrazides are also concerned in many other reactions. The non-alteration of the brain glutamate level has been taken to indicate the relative unimportance of this compound in such effects. However, when considering the possible action of these amino acids upon neurones, it is necessary to recognize that it is not their total brain level, but their extracellular concentration which determines whether or not the neurones will be affected. If inhibition of glutamic decarboxylase resulted in an increase of both intracellular and extracellular glutamate, the latter could produce convulsive activity by a direct action upon neurones, both glutamate and gamma-amino-*n*-butyric acid being then consumed as substrates for respiration during this increased activity (Tower, 1956; McKahnn & Tower, 1959). The total brain glutamate concentration might thereby be maintained at a relatively constant value, whereas the GABA level falls not only because of the inhibition of its production from glutamate but also because of its consumption during the increased respiration. The results of an earlier investigation (Curtis *et al.* 1959*b*) make it extremely unlikely that GABA has any role as a regulator of neuronal activity, for its depressant action has been shown to be non-specific. Accordingly, it is suggested that a more rational explanation of hydrazide convulsions lies in an increased extracellular excitant amino-acid concentration.

It has been possible to account for the convulsant action of both strychnine and tetanus toxin by the specific blocking action of these agents upon inhibitory synaptic transmission (Bradley, Easton & Eccles, 1953; Brooks, Curtis & Eccles, 1957). However, some convulsants have no such effect upon inhibition (Eccles, 1957; Curtis, 1959) and the action of these substances may be connected with disturbances of extracellular amino-acid concentrations, due to the inhibition of enzymes responsible for the maintenance of normal levels. It is pertinent in this respect that metrazol specifically sensitizes the cerebral cortex of the rabbit to the production of spreading depression by either glutamic or aspartic acids, a finding which led Van Harreveld (1959) to similar conclusions.

SUMMARY

1. Aspartate, glutamate and cysteate ions have been applied iontophoretically to the external surface membrane of neurones within the spinal cord of cats lightly anaesthetized with pentobarbital sodium.

2. These ions excite interneurones, Renshaw cells and motoneurones

by producing a membrane depolarization which, if of sufficient magnitude, initiates a spike potential.

3. It is considered that this action is non-specific and unrelated to excitatory synaptic transmitter action.

4. The findings are discussed in relation to the presence of these substances in the nervous system and to their role in convulsive disorders.

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THE EFFECTS OF HYDROCORTISONE ON THE LEVEL OF CORTICOTROPHIN IN THE BLOOD AND PITUITARY GLANDS OF ADRENALECTOMIZED AND OF STRESSED ADRENALECTOMIZED RATS

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The secretion of adrenocorticotrophin (corticotrophin, ACTH) appears to be controlled in several ways. The theory that its release from the adenohypophysis is regulated by the circulating level of corticoids was advanced by Sayers & Sayers (1947). These authors found that, in rats, the adrenocortical changes normally caused by stress could be prevented by previous treatment with corticoids. Several years previously Ingle, Higgins & Kendall (1938) showed that atrophy of the adrenal cortex followed the prolonged administration of adrenocortical extracts. Sayers & Sayers (1947) suggested that stress causes an increased utilization of corticoids by the peripheral tissues and the resulting low blood level of these hormones acts as a stimulus to the pituitary gland to increase its output of adrenocorticotrophin. Convincing evidence for the validity of such a 'peripheral humoral' theory came from demonstrations that animals with adrenocortical insufficiency exhibit high circulating levels of corticotrophin (Taylor, Albert & Sprague, 1949; Gemzell, Van Dyke, Tobias & Evans, 1951; Sydnor & Sayers, 1952).

However, many experimental results are difficult to reconcile with the existence of such a mechanism. The findings of Sayers & Sayers (1947) have been only partially confirmed. Some workers have found that the stress-induced secretion of ACTH can be prevented only by massive doses of corticoids (Hodges, 1953, 1954; Abelson & Baron, 1952) and others have failed to show any ability of these steroids to suppress ACTH release (Moya & Selye, 1948; Hall, Finerty, Hall & Hess, 1951; Fortier, Yrarrazaval & Selye, 1951). A further demonstration that the secretion of ACTH is not necessarily controlled by changes in the level of corticoids in the blood was provided by Sydnor & Sayers (1954) when they found that adrenalectomized animals are still capable of secreting adrenocorticotrophic hormone in response to stress. In fact, the release of ACTH from

the pituitary gland occurs more readily in the adrenalectomized than in the intact animal (Hodges & Vernikos, 1959).

Thus it appears that the importance of a peripheral humoral mechanism controlling the secretion of ACTH is not as great as was originally thought. In this paper are described the results of some experiments in which investigations were made of the effect of hydrocortisone on the levels of corticotrophin in the blood and pituitary glands of adrenalectomized and stressed adrenalectomized rats. The experiments were done in an attempt to provide further information concerning the role of corticoids in the regulation of pituitary adrenocorticotrophic activity.

METHODS

The experiments were performed on approximately 800 albino Wistar rats obtained from the Agricultural Research Council, Compton, Newbury. The animals were kept in a room, where the experiments were performed, at a constant temperature of 22° C for at least 1 week before being used. They were fed on a diet of cubes (diet 41, Lane-Petter & Dyer, 1952) and water.

Hydrocortisone was injected subcutaneously as suspension of 17-hydroxycorticosterone (Efcortelan, Glaxo) and intravenously as solution of hydrocortisone sodium succinate (Efcortelan soluble, Glaxo) in normal saline. The suspensions were made to contain 1, 5 and 15 mg hydrocortisone/ml. and the solutions to contain the equivalent of 2 or 8 mg hydrocortisone/ml. In acute experiments all hydrocortisone injections were made in volumes of 0·5 ml./100 g and in long-term experiments 0·1 ml./100 g body weight.

Bilateral adrenalectomy was performed under ether anaesthesia and the adrenalectomized rats were maintained on the normal diet with 0·9% sodium chloride solution in place of drinking water.

In all the experiments stress consisted of exposing the animals to ether vapour until they were deeply anaesthetized.

Blood samples were collected and their adrenocorticotrophic activity was estimated as described by Hodges & Vernikos (1959).

Pituitary glands were removed as rapidly as possible from decapitated female animals by the technique of Rochefort & Saffran (1957). The excised pituitaries were weighed immediately and transferred to tubes containing (per pituitary) 0·5 ml. N/10 hydrochloric acid. The glands were ground well with the aid of a glass rod and a little sand. At least six pituitaries were pooled and extracted thus for every determination. The extracts were stored in the refrigerator for 24 hr. They were diluted with normal saline immediately before injection into the assay animals and assayed for corticotrophin content on hydrocortisone-treated male rats, as described by Cox, Hodges & Vernikos (1958). A lyophilized preparation of ACTH (Armour) dissolved in normal saline was used as the standard. A conventional 2+2 assay design was employed and the results and their fiducial limits ($P = 0{\cdot}95$) were calculated by standard methods. The results were expressed as m-u. ACTH/pituitary gland and m-u. ACTH/mg pituitary tissue.

RESULTS

The effect of hydrocortisone on the blood level of corticotrophin in adrenalectomized rats, which had been anaesthetized with ether $2\frac{1}{2}$ min previously, is shown in Fig. 1. Hydrocortisone hemisuccinate equivalent to 4 mg hydrocortisone/100 g body weight, or normal saline, was injected

into the exposed femoral vein and the rats were decapitated 5, 10 or 20 min after the application of stress. The blood of the non-stressed adrenalectomized rat, from which the adrenal glands had been removed 5 days previously, produced no adrenal ascorbic-acid depletion in the assay animals, i.e. it possessed no detectable adrenocorticotrophic activity.

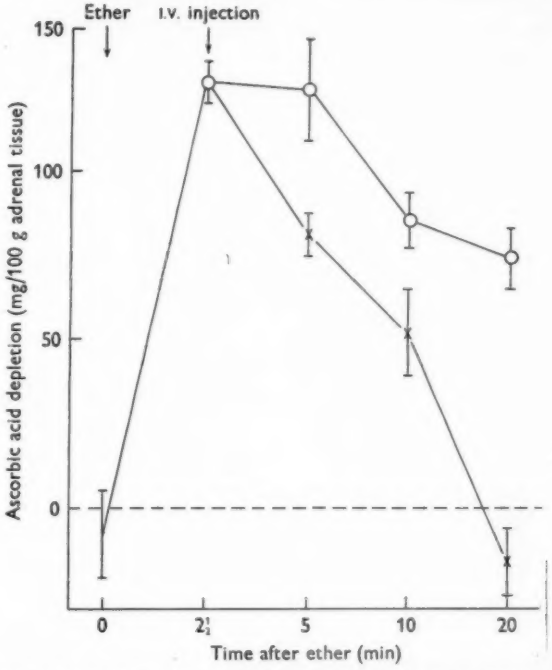

Fig. 1. Adrenal ascorbic acid depletions in assay rats after injections of blood collected from rats, adrenalectomized 5 days previously, at various times after stress (ether, first arrow). 2½ min after stress normal saline (○—○) or hydrocortisone 4 mg/100 g (×—×) was injected intravenously (second arrow). Vertical lines indicate standard errors.

Exposure to ether vapour caused a marked rise in circulating corticotrophin concentration to a readily detectable level. After the intravenous administration of normal saline this high level of circulating corticotrophin persisted for a few minutes and then gradually declined, as previously described by Hodges & Vernikos (1959). However, the intravenous injection of hydrocortisone caused a marked increase in the rate at which adrenocorticotrophic hormone disappeared from the blood. The activity fell to the pre-stress non-detectable level within 20 min.

Figure 2 shows the results of a similar experiment done on rats which had

been adrenalectomized 30 days previously. The blood of these animals, before subjection to stress, possessed considerable adrenocorticotrophic activity. This was raised further by exposure to ether. Normal saline, administered intravenously, did not alter the pattern of the usual fall in circulating corticotrophin to the pre-stress level. Hydrocorticone rapidly reduced the adrenocorticotrophic activity of the blood *but only to the pre-stress level.* The effect of 4 mg hydrocortisone/100 g was similar to, but more marked than, that of 1 mg of the steroid.

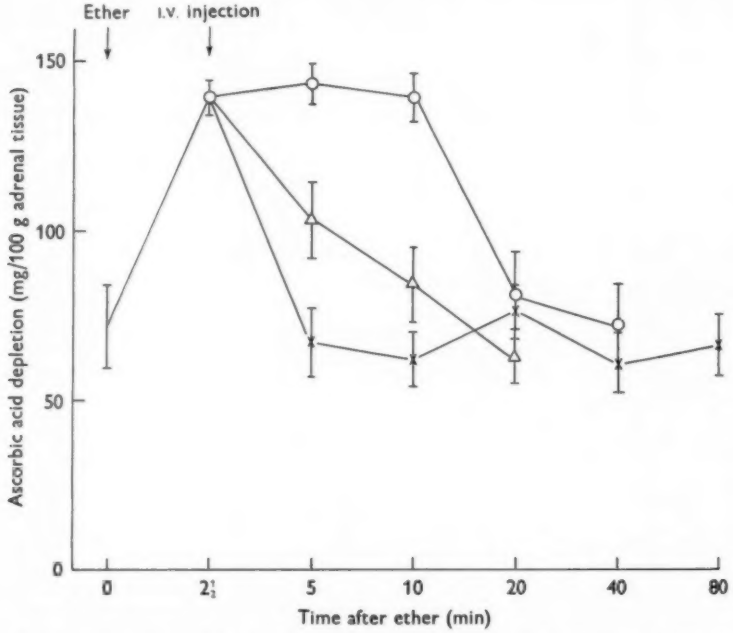

Fig. 2. Adrenal ascorbic acid depletions in assay rats after injections of blood collected from rats, adrenalectomized 30 days previously, at various times after stress (ether). 2½ min after stress normal saline (○—○) or hydrocortisone (4 mg/100 g, ×—× ; 1 mg/100 g, △—△) was injected intravenously. Each point represents the mean result obtained by injecting blood from 9 to 12 stressed animals into groups of 6 assay rats. The vertical lines indicate standard errors.

Figure 3 illustrates the results of an experiment in which the effect was studied of previous treatment with hydrocortisone on the level of corticotrophin in the blood of adrenalectomized rats before and after exposure to ether vapour. The animals were given subcutaneous injections of hydrocortisone (7·5 mg/100 g) or saline 4 hr before, and decapitated 2½ min after the stress. The saline-treated control animals, which had been adrenal-

ectomized 5 days previously, possessed no detectable level of circulating ACTH, in contrast to control rats adrenalectomized 30 days previously which exhibited high levels. Exposure to ether caused marked increases in the concentration of adrenocorticotrophic hormone in the blood of both groups of controls. Previous treatment with hydrocortisone prevented completely the stress-induced rise in circulating hormone but did not alter the resting level of corticotrophin in the blood.

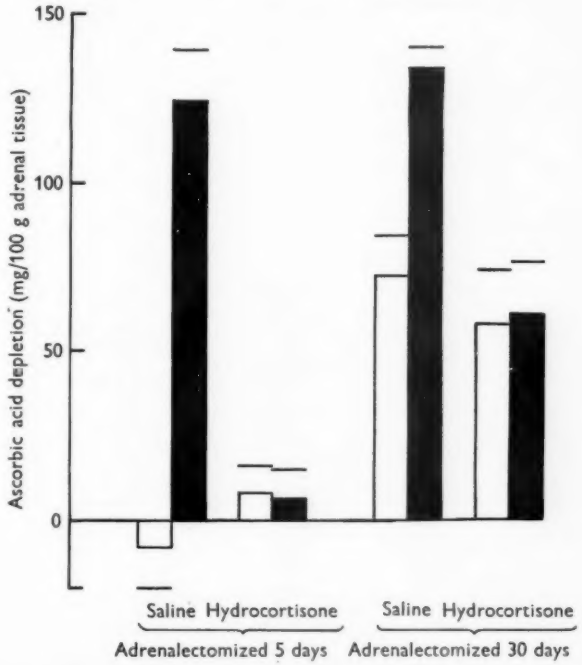

Fig. 3. Adrenal ascorbic acid depletions in assay rats after injections of blood from rats adrenalectomized 5 and 30 days previously. Donor rats were given subcutaneous injections of normal saline or hydrocortisone (7·5 mg/100 g) 4 hr before stress and the blood samples were collected before (non-stressed, □), or 2½ min after, exposure to ether (stressed, ■). Each column represents the mean result obtained by injecting pooled blood from 9 to 12 donor animals into groups of 6 assay rats. The horizontal lines above the columns indicate the standard errors.

Pituitary glands were obtained from intact rats, from animals 5, 10, 20, 30 and 50 days after adrenalectomy, and from rats adrenalectomized 30 days previously and given subcutaneous injections of hydrocortisone every day after the operation. After adrenalectomy the concentration of adrenocorticotrophic hormone in the pituitary gland fell slightly but

subsequently increased markedly. Thirty to fifty days after the operation the pituitary ACTH content was about $2\frac{1}{2}$ times the control level. The rise in pituitary corticotrophin content was completely prevented by daily treatment of adrenalectomized animals with 0·1 or 0·5 mg hydrocortisone. These results are shown in Table 1.

TABLE 1. Concentrations of adrenocorticotrophic hormone in the pituitary glands of normal intact, adrenalectomized and adrenalectomized-hydrocortisone-treated rats. Fiducial limits ($P = 0{\cdot}95$) given in parentheses

Experimental condition	No. of rats	Mean rat weight (g) ± S.E.	Mean pituitary weight (fresh) (mg) ± S.E.	No. of assay rats	Corticotrophin concentration: m-u./pituitary gland (and range)	Corticotrophin concentration: m-u./mg pituitary tissue	Corticotrophin concentration: % of control
Normal, intact (controls)	6	167 ± 4	8·3 ± 0·6	12	129 (92–177)	15·5	—
	6	140 ± 3	7·1 ± 0·4	12	84 (50–142)	12	—
Adrenalectomized 5 days	6	145 ± 2	8·1 ± 0·6	10	89 (53–207)	11	83
10 days	6	147 ± 4	8·6 ± 0·3	10	128 (97–170)	14·9	120
20 days	6	154 ± 4	8·6 ± 0·3	12	220 (154–314)	25·6	205
30 days	6	169 ± 6	9·3 ± 0·4	12	288 (190–436)	30·1	266
50 days	5	186 ± 7	9·5 ± 0·6	12	280 (166–473)	30	259
Adrenalectomized 30 days + 0·1 mg hydrocortisone s.c. per day	6	155 + 3	7·6 ± 0·2	12	110 (75–161)	14·5	103
	6	140 ± 7	8·1 ± 0·5	12	92	11·3	86
Adrenalectomized 30 days + 0·5 mg hydrocortisone s.c. per day	6	124 ± 5	7·3 ± 0·5	12	92 (54–154)	12·6	86

Figure 4 summarizes the results of experiments in which the blood corticotrophic activity was estimated before and after stress in hydrocortisone-treated and untreated rats 30 days after adrenalectomy. The untreated animals developed a high circulating ACTH level which was raised further by stress; hydrocortisone in daily doses of 0·1 or 0·5 mg almost completely prevented the development of this high circulating ACTH level. However, the rise in blood corticotrophin which occurred in the adrenalectomized animal after stress was not influenced by the smaller dose and only partially prevented by the larger dose of the steroid.

DISCUSSION

The facts that the adrenocorticotrophic activity of the pituitary gland can be suppressed by a high blood level of corticoids and that animals with adrenocortical insufficiency exhibit high levels of circulating cortico-

trophin provide the strongest arguments for the existence of a peripheral humoral mechanism controlling the secretion of adrenocorticotrophic hormone. However, the arguments become less convincing when the high dosage of corticoids necessary to suppress the pituitary gland, and the long delay in the development of increased blood corticotrophin levels after adrenalectomy, are taken into consideration. Certainly, the precise function of corticoids in regulating pituitary adrenocorticotrophic activity is still not established. The present series of experiments appears to provide further information concerning their possible role.

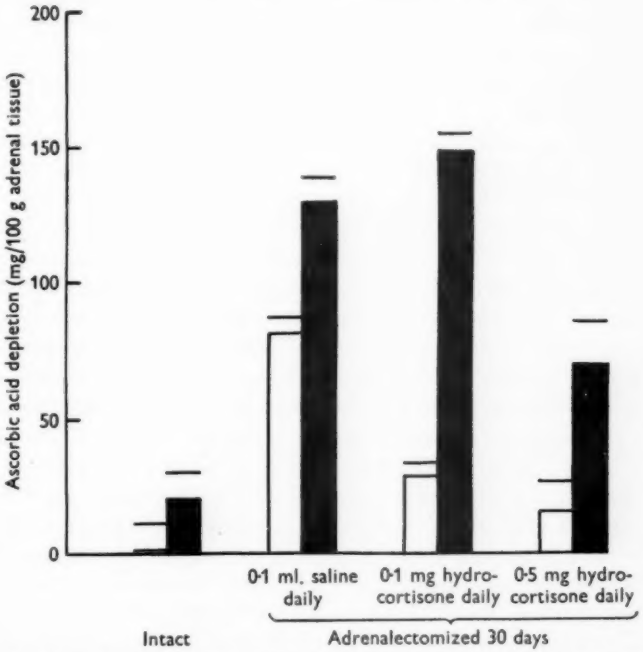

Fig. 4. Adrenal ascorbic-acid depletions in assay rats after injections of blood from stressed- (■) and non-stressed (□) rats, adrenalectomized 30 days previously, and given daily subcutaneous injections of normal saline or hydrocortisone. Conventions as in Fig. 3.

Previous work done in this laboratory showed that the adrenalectomized rat did not develop an elevated, fairly constant and easily detectable concentration of circulating adrenocorticotrophin until 3–5 weeks after adrenalectomy (Cox *et al.* 1958). This high concentration of the hormone in the blood could be raised even further by stress (Hodges & Vernikos, 1959). The present findings indicate that the administration of a

single large dose of hydrocortisone to adrenalectomized rats does not affect the resting level of ACTH in the blood but completely prevents the stress-induced rise in circulating hormone. Thus the high blood level of ACTH which occurs 20–30 days after adrenalectomy could not be reduced by hydrocortisone but the further rise normally caused by stress could be completely prevented. Similarly, intravenous administration of hydrocortisone, at the time when the maximum blood level of corticotrophin is attained after stress, rapidly reduces the circulating hormone concentration but again *only to the pre-stress level*. These observations suggest that hydrocortisone does not interfere with the release of corticotrophin by acting directly on the pituitary gland. However, it can prevent the increased pituitary adrenocorticotrophic activity caused by stress. It may act by interfering with the secretion of corticotrophin releasing factor as has been suggested by McCann, Fruit & Fulford (1958). This action of hydrocortisone is produced only with large doses of the steroid and is probably not of physiological significance.

The increase in pituitary corticotrophin content which occurs after adrenalectomy has been demonstrated previously (Sydnor & Sayers, 1954; Fortier, 1959). It can be prevented by daily treatment of adrenalectomized animals with doses of hydrocortisone which are far too small to influence the release of corticotrophin in response to stress. The fact that this effect can be produced by such small doses of hydrocortisone suggests that the corticosteroids are concerned with the control of the rate at which adrenocorticotrophin is synthesized and stored in the pituitary gland rather than with the release of the hormone. Recently Fortier & de Groot (1959) also came to the conclusion that the regulating effect of corticosteroids is predominantly on corticotrophin synthesis. They based their conclusion on the results of experiments in which they correlated the changes in pituitary corticotrophin level with those in plasma corticosteroid concentration in rats with one adrenal removed and the other enucleated. The pituitary ACTH content was elevated markedly during the period of adrenal insufficiency, but then declined as the cortical tissue of the enucleated adrenal gland regenerated and the corticosteroid level in the blood increased. Their observations are consistent with those of Farrell & Laqueur (1955) who demonstrated a fall in pituitary corticotrophin content in dogs given large daily doses of cortisone.

Dosing adrenalectomized rats with only 0·1 mg hydrocortisone per day was sufficient to prevent the increase in pituitary corticotrophin content. This dose was considered to be near the physiological maintenance dose since the treated adrenalectomized animals, in contrast to untreated animals and those which received larger daily amounts of the steroid, continued to grow well and were in excellent condition. However, when

subjected to stress at the end of the experimental period they exhibited a marked increase in the level of circulating corticotrophin. In this respect they resembled rats from which the adrenals had been removed a few days previously, and not normal intact animals (Hodges & Vernikos, 1959). Thus adrenalectomy appears to result in increased excitability of the adenohypophysis, which is not reversed by treatment with maintenance doses of hydrocortisone. It is difficult to explain this observation but it is interesting to note, in this connexion, that McCann *et al.* (1958) suggested that corticoids partially influence the release of ACTH by depressing the excitability of the adenohypophysis. Our results appear to indicate that the increased excitability of the adenohypophysis of the adrenalectomized rat is not due to corticoid deficiency.

The time relationships of the changes in blood corticotrophin concentration which occur after adrenalectomy have been described in previous papers from this laboratory (Cox *et al.* 1958; Hodges & Vernikos, 1959). There is a fairly close relationship between the rates at which the pituitary level and the blood concentration of ACTH increase after the operation. Cox *et al.* (1958) suggested that the delay in the development of high levels of circulating ACTH was due to the slow rate at which the pituitary gland could adapt itself to secrete and maintain an enormously increased output of ACTH in response to the complete absence of circulating corticoids. A more satisfactory explanation is that the low plasma corticoid level results in increased synthesis of ACTH in the pituitary gland and that the rise in circulating ACTH is due to hormone 'spill over' from the increased pituitary stores.

The release of ACTH from the adenohypophysis *can* be suppressed, but only by very high, non-physiological, blood levels of corticoids. High levels of circulating adrenocorticotrophic hormone *do* occur in animals exhibiting adrenocortical insufficiency but only after the pituitary stores of the hormone have increased considerably. Therefore it is unlikely that the secretion of ACTH is controlled by alterations in the blood corticoid level. There can be no doubt, however, that corticoids play some part in regulating the adrenocorticotrophic activity of the anterior lobe. The results of the experiments described in this paper add further support to Fortier's suggestion that their action is predominantly on the synthesis of corticotrophin.

SUMMARY

1. Corticotrophin was not detectable in the blood of rats, which had been adrenalectomized 5 days previously, before exposure to ether (stress), but was readily detectable 2½ min afterwards. Thirty days after adrenalectomy rats exhibited a high resting blood corticotrophin level which was raised even further by stress.

2. Hydrocortisone, administered intravenously in large doses to adrenalectomized rats 2½ min after stress, rapidly reduced the resulting high concentration of circulating corticotrophin, but only to the pre-existing level.

3. Previous treatment of adrenalectomized rats with large doses of hydrocortisone did not affect the resting blood level of corticotrophin but prevented the stress-induced rise in circulating hormone.

4. Adrenalectomy resulted in a slight fall and then a marked rise in pituitary corticotrophin content. This rise was prevented by daily treatment with small doses of hydrocortisone.

5. Similar doses of hydrocortisone almost completely prevented the rise in circulating corticotrophin which occurred after adrenalectomy, but did not prevent the development of markedly elevated blood levels of the hormone in response to stress.

6. The results add further support to the hypothesis that the action of corticoids in controlling pituitary adrenocorticotrophic activity is mainly on corticotrophin synthesis.

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A COMPARATIVE STUDY OF FOUR MEANS OF EXPRESSING THE METABOLIC RATE OF RATS

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The oxygen consumption of rats is frequently used as an indication of heat production. Before we can draw conclusions regarding the effects of any procedure on the heat production of the animals it is necessary to eliminate differences arising from age, sex and weight. By using the animals as their own controls or by using control groups of the same age and sex differences due to these factors are removed. The procedure to be tested may, however, lead to changes in weight or in growth rate of the test animals. These changes may be sufficient to result in differences in weights of the test and control groups. Hence it is not often possible to follow the advice of Benedict (1938) to compare the metabolism of animals having the same weight. The usual practice has heen to refer the absolute oxygen consumption to some unit which supposedly corrects for body weight or size. While the situation has improved somewhat since Kleiber (1944) first expressed alarm, four reference units are still in general use. These are: body weight (kg), 'metabolic size' ($kg^{\frac{3}{4}}$) or ($kg^{\frac{2}{3}}$) and surface area (m^2 calculated from body weight). Since there can be only one correct way of expressing oxygen consumption and since conclusions often depend upon the assumption that the reference unit used is the proper one, it is surprising that no comparative study of the four means of expressing heat production has been made. This paper reports the results of such a study.

METHODS

Male albino rats were used for all experiments. They were kept in individual metal cages and fed on Purina Laboratory Chow and water *ad libitum*.

Oxygen consumption was recorded for 1 hr by a closed-circuit method previously described (Hsieh & Chiu, 1959). Six units were used, making possible twelve tests each morning. During the tests the air surrounding the animals was maintained at about 25° C.

In one series of experiments the ages of the rats were ignored. Ninety animals of different weights were obtained from the animal house and kept at 28° C for about 5 days. Oxygen consumption was determined after this period. This experiment was designed to imitate the usual procedure, in which rats of different ages and weights are used.

To test the effects of age on oxygen consumption and to obtain rats of the same age but with different weights the following experiments were performed. Thirty-three rats about 7 weeks old were divided into three groups of eleven each. One group (*WN*) was kept at

28° C. The second group (*WT*) was kept at 28° C and deprived of both food and water one day a week. The third group (*C*) was kept at about 6° C.

To produce short-term changes in weight a group of six rats was deprived of both food and water for 2 days. Another group of six rats was given a single subcutaneous injection of 0·5 mg reserpine (Serpasil, Ciba). Rats of these groups were maintained at 28° C.

Surface area was calculated from body weight (g) by the formula: Surface area (m^2) = $0{\cdot}0011(g^{0{\cdot}63})$ (Kibler & Brody, 1942). For convenience we have expressed surface area in units of 0·1 m^2.

RESULTS

The absolute rates of oxygen consumption of ninety rats are plotted against their body weights in Fig. 1. If we assume linear regression, basing absolute oxygen consumption on surface area yielded the highest correlation coefficient (Table 1). However, the line best fitting the results is of the type: $Y = a + bX + cX^2$ (second degree polynomial), where X represents body weight in kg. The correlation coefficient of this line is significantly

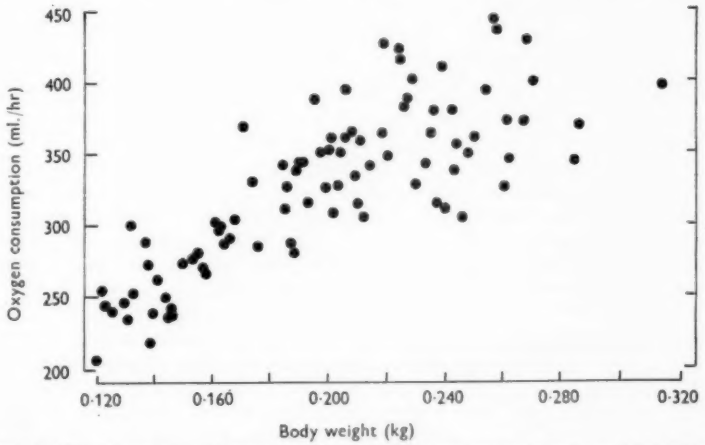

Fig. 1. Oxygen consumption of ninety rats plotted against their body weights. The rats were kept at about 28° C and oxygen consumption measured for 1 hr at about 25° C.

higher than that of the others. When the oxygen consumption of rats weighing over 200 g is expressed in terms of body weight, 'metabolic size' or surface area there is a significant negative regression with body weight (Fig. 2). The average hourly oxygen consumption of the rats weighing over 200 g is shown in Table 2.

The comparative rates of growth of the *WN*, *WT* and *C* rats are shown in Fig. 3. The rats which fasted recovered their weights on the day after the fast and then grew at about the same rate as the group which did not fast. Thus the *WT* group lost about 2 days of growth per week and at

the age of 19 weeks, after 11 days of weekly fasting, they weighed the same as the *WN* rats at 15 weeks of age. At 19 weeks the group kept at 6° C weighed the same as the *WN* group at 12 weeks of age.

The change in metabolic rate with age of the *WN* group is shown in Fig. 4. There was a gradual decrease in metabolic rate with increasing age. A statistical analysis of the data indicates, however, that the change

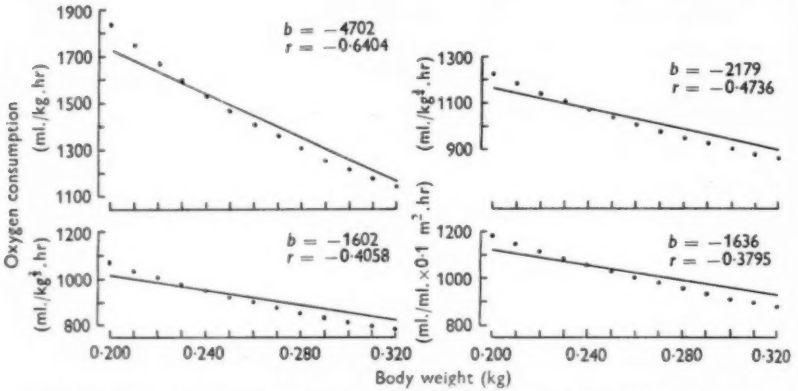

Fig. 2. Regression of metabolic rates, expressed in various units, of forty-six rats. To simplify calculations linear regression was assumed. The negative slopes, *b*, are all significant at the 2% level. The dots indicate rates calculated by assuming a fixed consumption of 368 ml./hr. Expressing the oxygen consumption of rats weighing more than 200 g in terms of their body weight, 'metabolic size' or surface area does not correct for differences in body weight.

TABLE 1. Regression equations of oxygen consumption of rats on their body weights (kg), 'metabolic sizes' ($kg^{⅔}$ and $kg^{¾}$) and surface areas ($0{\cdot}1\ m^2$)

Reference unit (X)	Experimental range of X	Regression equation Y = O_2 consumption (ml./hr)	S.E. of estimate	Correlation coefficient
kg	0·120–0·314	$Y = 3341X - 5899X^2 - 92{\cdot}4$	30·8	0·8344
kg	0·120–0·314	$Y = 133 + 972{\cdot}4X$	33·3	0·8001
$kg^{⅔}$	0·204–0·419	$Y = 68{\cdot}4 + 872{\cdot}9X$	32·8	0·8076
$kg^{¾}$	0·243–0·462	$Y = 36{\cdot}4 + 856{\cdot}8X$	32·7	0·8084
$0{\cdot}1 m^2$	0·225–0·413	$Y = 20{\cdot}1 + 999{\cdot}1X$	32·6	0·8103
	$n = 90$			

TABLE 2. The oxygen consumption of a group of forty-six rats with average weight 236 g (S.D. 25·7 g)

Hourly rate of oxygen consumption	S.E.	Coefficient of variation (%)
367·7 ml.	4·94	9·2
1558 ml./kg	27·8	12·1
1082 ml./$kg^{⅔}$	17·4	10·9
958·3 ml./$kg^{¾}$	15·0	10·6
1064 ml./0·1 m^2	16·4	10·4

is due to increasing weight and not to age. This fact is evident from inspection of Fig. 5.

The relative rates of increase in body weight and absolute oxygen consumption of the *WN*, *WT* and C rats are shown in Fig. 6. To enable comparison these are expressed as percentages, with body weight and oxygen consumption at 7 weeks taken as 100 %. The relative rates of increase in body weight and oxygen consumption are different in the different groups.

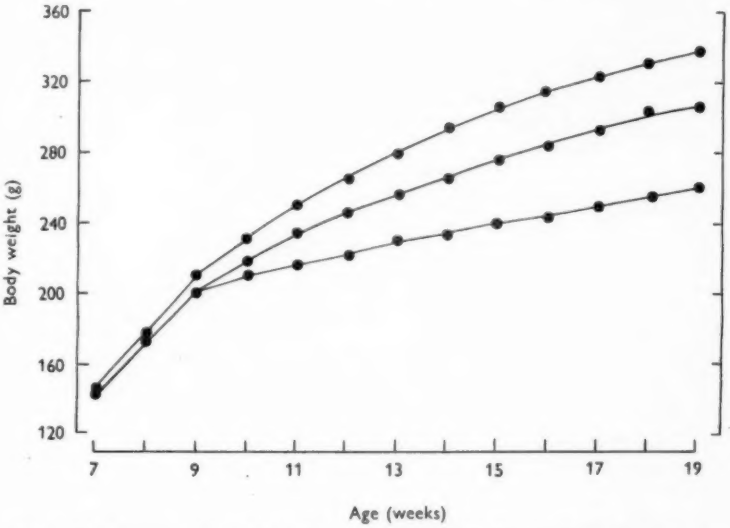

Fig. 3. The growth rates of rats from 7 to 19 weeks of age. Upper curve, rats kept at 28° C (*WN*). Middle curve, rats kept at 28° C and deprived of food for 1 day a week beginning with the eighth week (*WT*). Lower curve, rats kept at 6° C (*C*). Each group consisted of eleven rats.

The oxygen consumption of the C rats unfortunately was not determined between the eighth and thirteenth week. The results for the last 5 weeks of the experiment suggest that the absolute oxygen consumption of this group may have increased at a more rapid rate than those of the other two groups. At 19 weeks of age no significant difference existed in the oxygen consumption of the three groups. There was a difference in their metabolic rates (Table 3). However, the mean metabolic rates of the three groups, adjusted for differences in weight, were not significantly different.

The effects of a two-day fast and of reserpine on the body weight and oxygen consumption are shown in Figs. 7 and 8. On the second day of the fast body weights had fallen by about 39 g and oxygen consumption

by about 89 ml./hr. These represent reductions of about 18 and 26%, respectively. The reduction in metabolic rates depends on the unit used, being about 11% when based on body weight and 17% when based on surface area. The injection of reserpine was followed by a reduction in oxygen consumption which persisted for about 3 days. On the first day after the injection of reserpine there were small, but significant, increases in metabolic rates.

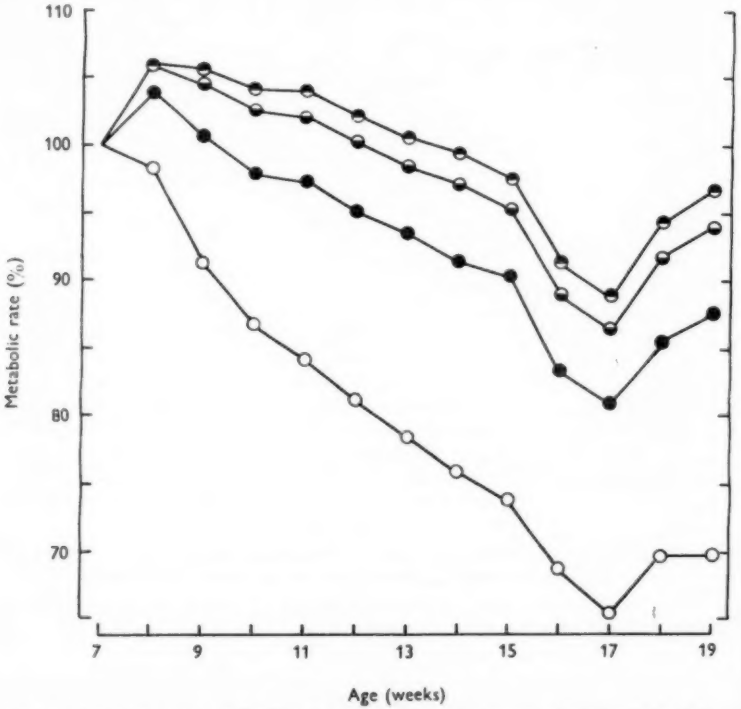

Fig. 4. Metabolic rates of *WN* rats, expressed as percentages, plotted against their ages in weeks. The metabolic rate at 7 weeks was taken as 100%. ○, rate in ml./kg.hr; ●, rate in ml./kg$^{\frac{3}{4}}$.hr; ◒, rate in ml./kg$^{\frac{3}{4}}$.hr; ◓, rate in ml./0·1 m^2.hr.

DISCUSSION

The term 'metabolic rate' is poorly defined. Kleiber (1956) uses it to mean either the absolute rate of heat production or the rate of heat production per unit of body size. We feel that a distinction between these two meanings is necessary. In experiments on fasting rats, Rixon & Stevenson (1957) observed that metabolic rate based on absolute weight showed better correlation to weight loss during fasting than when based

on $kg^{\frac{3}{4}}$ units or on surface area. However, the linear regression of pre-starvation metabolic rate on the rate of weight loss during fasting was obtained by plotting oxygen consumption, in ml./min/100 g pre-fasting body weight, against loss of weight in g/day/100 g pre-fasting body weight. Hence the absolute rate of oxygen consumption was plotted against the absolute rate of weight loss. These authors also obtained a linear regression

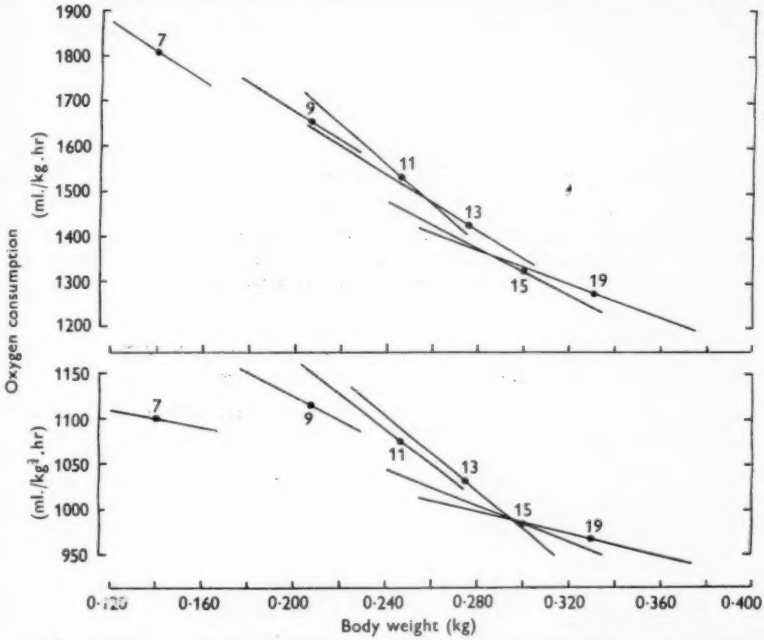

Fig. 5. The regression of metabolic rate on body weight of *WN* rats. Upper, rate in ml./kg.hr; lower, rate in ml./$kg^{\frac{3}{4}}$.hr. The dots represent mean values and the nearby numbers the ages in weeks of the rats.

when they plotted the ratio, total weight loss (g/100 g pre-fasting weight): pre-fasting oxygen consumption (ml./100 g pre-fasting weight), against survival time. Here again absolute values were employed. Indeed, if $kg^{\frac{3}{4}}$ units, or any other unit, had been used to express both rates of oxygen consumption and weight loss similar results would have been obtained. These facts are pointed out not to criticize the conclusions of Rixon & Stevenson (1957) but to show that the free use of the term 'metabolic rate' can be misleading. In the present discussion 'oxygen consumption' is used to indicate the rate of oxygen consumption per rat and 'metabolic rate' is limited to those observations in which the absolute rates have been based on some unit of size.

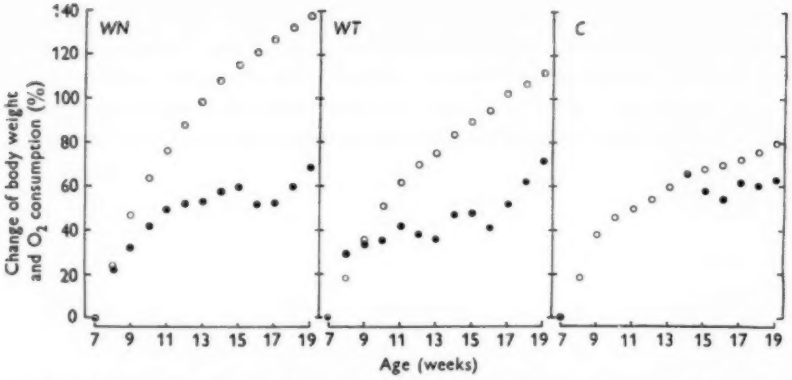

Fig. 6. The relative rates of increase in body weight and oxygen consumption of *WN*, *WT* and *C* rats. Body weight and oxygen consumption at 7 weeks of age are taken as 100%. ○, body weight; ●, oxygen consumption.

TABLE 3. Oxygen consumption of warm-adapted rats (*WN*, *WT*) and cold-adapted rats (*C*) 19 weeks of age

Group	No.	Average weight (g)	Average hourly oxygen consumption ml.	ml./kg
WN	11	346 ± 4·8%	434 ± 7·7%	1254 ± 6·3%
		$P < 0·001$	N.S.	$P < 0·001$
WT	11	299 ± 7·2%	428 ± 6·0%	1436 ± 7·2%
		$P < 0·001$	N.S.	$P < 0·01$
C	11	260 ± 5·7%	412 ± 6·6%	1580 ± 6·0%
WN + *WT*	22	322 ± 9·5%	431 ± 6·8%	1345 ± 9·6%
WN + *WT* + *C*	33	301 ± 13·1%	425 ± 7·1%	1423 ± 11·4%

Group	No.	Average hourly oxygen consumption ml./kg$^{\frac{3}{4}}$	ml./kg$^{\frac{1}{3}}$	ml./0·1 m^2
WN	11	962 ± 6·3%	880 ± 6·4%	992 ± 6·4%
		$P < 0·01$	$P < 0·01$	$P < 0·01$
WT	11	1063 ± 6·0%	960 ± 5·8%	1074 ± 5·8%
		$P < 0·05$	$P < 0·05$	$P < 0·05$
C	11	1130 ± 5·6%	1040 ± 5·5%	1130 ± 5·5%
WN + *WT*	22	1012 ± 7·9%	920 ± 7·4%	1033 ± 7·2%
WN + *WT* + *C*	33	1051 ± 8·8%	960 ± 8·2%	1065 ± 7·9%

± = coefficient of variation

Horst, Mendel & Benedict (1934) noted that the metabolic rates of their rats were not in accord with an earlier study. As the weights of the two groups of rats differed widely these authors suggested that the lack of agreement was due to the fact that heat production expressed per unit of weight or per unit of surface area did not rule out the difference in body weight. Our results clearly show that there is a negative regression of metabolic rate on body weight (Figs. 2 and 5). Therefore, when comparing the metabolic rates of groups of animals with different average body weights,

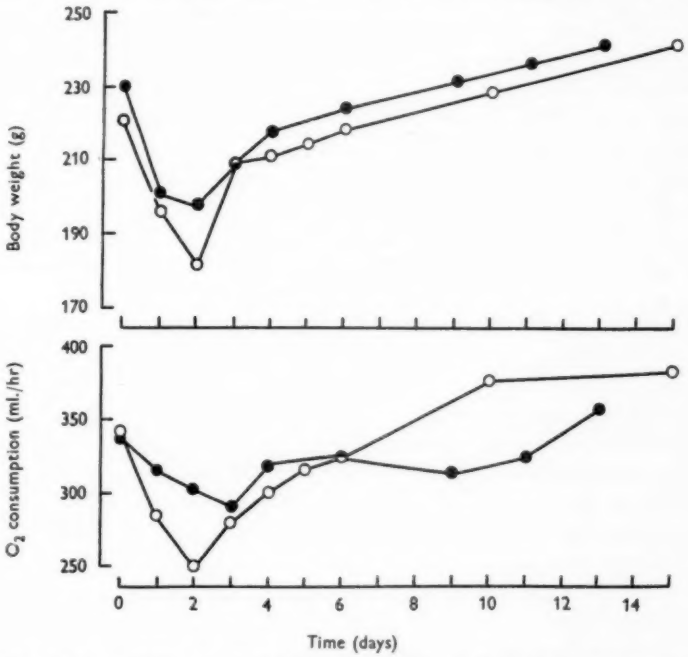

Fig. 7. The effects of a two-day fast, ○, and of a single subcutaneous injection of 0·5 mg reserpine, ●, on the body weight and oxygen consumption of rats. Each point represents the average from six rats.

it is necessary to perform an analysis of co-variance and to seek differences in the adjusted mean metabolic rates. If these steps are not taken, conclusions will depend upon the degree of difference in body weight and on the reference unit used, since the regression coefficients are different for the different reference units. Benedict & Sherman (1937) have pointed out that the metabolic rate of old rats is higher than middle-aged ones when based on body weight but not when based on surface area. Black & Murlin (1938) observed that the metabolic rate of rats falls gradually

during the first 6 months of life. Figure 4 shows that our results indicate the same relationship. If it is assumed that corrections for changes due to weight have been made by using metabolic rate then one may conclude that the gradual reduction of metabolic rate is due to age. But age does not affect the metabolic rate (Fig. 5). Another example of erroneous

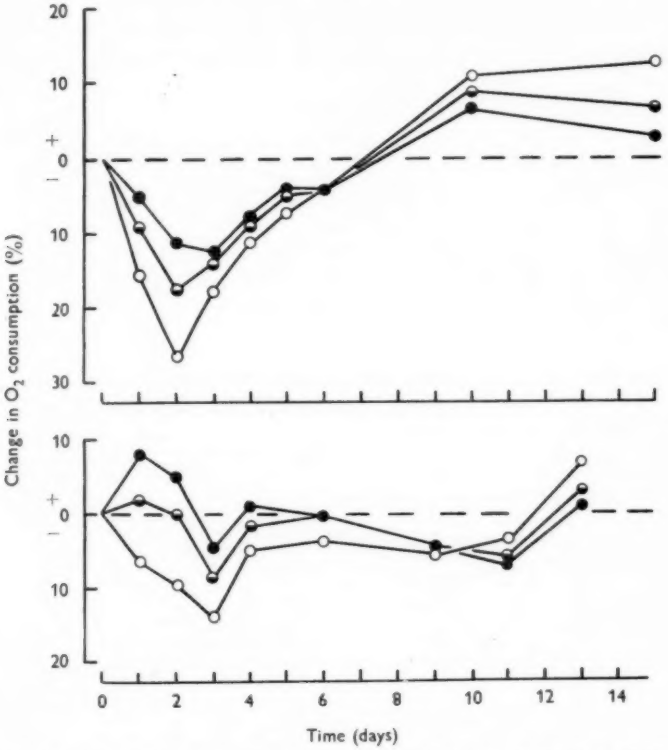

Fig. 8. Upper, change in oxygen consumption of rats deprived of food for 2 days; lower, after receiving a single subcutaneous injection of 0·5 mg reserpine. Oxygen consumption is expressed in ml./hr, ○; ml./kg.hr, ●; ml./0·1 m^2.hr, ◒. Each point represents the average from six rats.

conclusions arising from the assumption that metabolic rates correct for weight is shown in Table 3. The metabolic rates of the moderately fasted group (*WT*) are significantly higher than those of the normal controls (*WN*). In the present experiments one might conclude that this was an effect of moderate fasting. Without knowing that the *WT* group had been deprived of food one might conclude, of course, that the rats in the *WT* group were thinner because of their higher metabolic rate. There is, how-

ever, no difference in the metabolic rates of the two groups when these are adjusted for body weight.

One of the reasons for basing oxygen consumption on body size is the desire to reduce the coefficient of variation of the results. When the range of body weights of the animals is small not much difference is found between the coefficients of variation of oxygen consumption and metabolic rates. However, when the range of body weights is large, expressing oxygen consumption in terms of a unit of size increases the coefficient of variation (Table 2 and 3). From this it follows that if an analysis of co-variance is not performed on the results, comparing the absolute oxygen consumption of rats of the same age but of different weights will introduce less error.

Another reason advanced for using metabolic rates is the desire to predict absolute heat production from body size (Kleiber, 1947). Kleiber (1956) offers the formula: $M = 3(\text{body weight in kg}^{\frac{3}{4}})$; where M is the heat production in kcal/hr. This formula is only an approximation and Kleiber suggests that $M = k(\text{body weight in kg}^{\frac{3}{4}})$ be used for more accurate work. Since k has to be calculated from the absolute heat production and is different for different body weights we fail to see the usefulness of this formula.

Kleiber (1947) points out that considerations of heat transfer do not support the hypothesis that the heat production of homoeotherms should be strictly proportional to surface area. He does not say in what way these considerations support his hypothesis that heat production should be proportional to the body weight in $kg^{\frac{3}{4}}$ units. The $kg^{\frac{3}{4}}$ unit was chosen in order to make the metabolic rates of homoeotherms, from mice to elephants and whales, approximately the same. In studies on temperature regulation the reference of heat loss to surface area is theoretically correct. It should be noted, however, that the surface area of rats, and of other laboratory animals, is really an expression of weight, since it is calculated from body weight and the constant is merely for the sake of units.

The oxygen consumption of an animal at rest and at its 'critical' environmental temperature increases with age, just as the actual body weight increases with age, but the relative rates of increase of the two are different (Fig. 6). This can be explained by assuming that the oxygen consumption depends upon the 'activity' and amount of metabolically active tissue while the actual body weight depends upon the amount of metabolically active tissue plus inert material (e.g. fat and water). It is conceivable that during different stages of development the 'activity' and the rate of production of metabolic material are different. In addition, the rate of production of inert material may also vary. These considerations make it illogical to expect a constant relationship between oxygen consumption and body weight, or any power of body weight, throughout the

life of an animal. The relative rates of increase of oxygen consumption and of total body weight may also depend upon environmental (temperature) conditions and the nutritional (quantity and quality of food available) and hormonal status of the animal. For these reasons one cannot expect the ratio oxygen consumption: body size to be the same in normal animals and, say, in animals kept in a cold environment. The proper unit for comparing the metabolic activity of the tissues of animals is the ratio oxygen consumption: size of tissues involved. The use of lean body mass as an indication of metabolic size has been recommended (Keys & Brožek, 1953). But the estimation of lean body mass of rats is often not practical, as all procedures, with the exception of the one proposed by Lesser, Blumberg & Steele (1952), involve the death of the animals. With small animals the use of specific gravity as an indication of fat content results in large errors of determination which cancel the theoretical advantages from the use of this unit. Da Costa & Clayton (1950) found good correlation ($r = -0{\cdot}63$) between body density and fat content in rats. However, Keys & Brožek (1953) have pointed out that the correlation coefficient was not high enough to permit precise individual predictions. The calculation of body fat content from specific gravity data is based on the principle of Archimedes and requires prior knowledge of the densities of the 'fat' and the 'lean' portions of the animal. At present the conversion of specific gravity to percentage of fat is based almost entirely on data obtained from the guinea-pig (Rathbun & Pace, 1945; Pace & Rathbun, 1945).

Our results suggest that it is safe to assume that mature rats of the same age have the same amounts of metabolically active tissue. Hence it is possible simply to use oxygen consumption as a direct indication of tissue activity. The results, however, hold only for rats that weigh between 250 and 350 g at 19 weeks of age. With greater discrepancies in mean body weights between two groups direct comparison may lead to errors and should not be undertaken. In such cases it is necessary to use both absolute oxygen consumption and oxygen consumption adjusted for weight for comparisons, assuming a specific action on energy metabolism to have occurred when both have changes in the same direction. The basis for this type of comparison is given by Heroux & Gridgeman (1958).

In experiments of a few days duration and where the animals are used as their own controls the problem of determining the effects of a procedure on energy metabolism is simpler. Here it can be assumed that changes with time in the amount of metabolically active tissue are small; thus any alteration in oxygen consumption may be ascribed to changes in the activity of the cells. Carr & Beck (1937), basing heat production on body weight, noted that there was no change in the metabolic rate of rats which fasted for 48 hr. Our results are not comparable since the starved rats of

the present experiments suffered both inanition and dehydration. However, the use of absolute oxygen consumption as an indication of tissue activity suggests that the effects of fasting are greater than have been assumed.

The results obtained 24 and 48 hr after the injection of reserpine suggest that this drug depresses the heat production of rats. A single large dose of reserpine in rats results in diarrhoea and marked loss of weight for about 2 days. This may be due to increased peristaltic activity (Bülbring & Crema, 1959). Hoffman (1958), basing oxygen consumption on body weight, obtained a 25–30 % increase in metabolic rate of rats 1–6 hr after the injection of reserpine. Only part of this increase can be ascribed to a decrease in weight of the animals. It would seem therefore that the short-term effects of reserpine lead to an increase in heat production, possibly due to vasodilatation and increased heat loss.

SUMMARY

1. A comparative study has been made of four means of expressing the metabolic rate of rats by determining their absolute oxygen consumptions and basing results on body weight (kg), 'metabolic size' ($kg^{\frac{2}{3}}$ and $kg^{\frac{3}{4}}$) and surface area (m^2) calculated from body weight.

2. Expressing the oxygen consumption of rats weighing more than 200 g in the above terms does not correct for differences that are caused by differences in body weight.

3. There is a gradual fall in metabolic rate of rats with increasing age from 7 to 19 weeks. This is due to increasing weight of the animals.

4. After 11 weeks of moderate fasting (removal of food and water one day each week) rats have higher metabolic rates than normal controls. This is due to the lower body weights of the fasted group.

5. Rats kept in a cold environment (6° C) have a higher metabolic rate than controls kept at 28° C. This is due to the lower body weights of the former group.

6. The shortcomings of the term 'metabolic rate' are discussed and reasons advanced why one cannot expect a constant relationship between oxygen consumption and body weight or any power of body weight.

7. It is suggested that, where discrepancies in the body weights of two groups are small, comparing absolute oxygen consumption will yield more accurate results. Where the difference in weight between two groups is greater than 100 g (the limits of the present experiments) it will be necessary to compare both absolute oxygen consumption and oxygen consumption adjusted for weight, regarding a specific action on energy metabolism as having taken place when both have changed in the same direction.

8. By the above method it is concluded that acute fasting (removal of food and water for 48 hr) has a greater depressing effect on energy metabolism than can be presumed from the calculated metabolic rate. This method also indicates that reserpine has a depressing effect on heat production.

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SPONTANEOUS ACTIVITY IN MUSCLE FIBRES OF THE CHICK

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It has recently been reported (Ginsborg, 1959) that muscle fibres of the chick are innervated in one of two different ways. One type of fibre is supplied by a single axon and has a focal end-plate. The other type is supplied by a number of axons and has neuromuscular junctions distributed at many points along the fibre. The evidence for this view was derived, in part, from the two different kinds of spontaneous electrical activity which could be recorded from within single fibres of a chick's muscle, and it is the purpose of this paper to describe that spontaneous activity in greater detail. The two types of activity may conveniently be referred to as 'focal' and 'diffuse'.

METHODS

Intracellular recording from single fibres of various muscles of the chick was employed. The micro-electrodes, usually filled with 5M-NaCl, and the experimental arrangements were conventional (Fatt & Katz, 1951). In some experiments two electrodes were inserted into the same fibre, the second electrode being used either as a polarizing or as an additional recording electrode. The muscles were removed from chicks of from 1 day to 6 weeks in age anaesthetized with 2 ml./kg of a 9% solution of Na-phenobarbitone (I.V.), and mounted in a chamber similar to that described by Boyd & Martin (1956), in which the bathing fluid was circulated by a gas lift (O_2 95%, CO_2 5%). The composition of the bathing fluid was: (mM) NaCl 150, $NaHCO_3$ 20, KCl 5·0, $CaCl_2$ 5·0, $MgCl_2$ 2·0, with glucose 2 g/l.; the concentrations of cations were based on the analyses of cock plasma given by Lake, Butler, McCallum & MacIntyre (1958). The solution described by Krebs & Henseleit (1932) appeared equally satisfactory. In some experiments solutions were made hypertonic with NaCl in order to increase the frequency (which varied between about 1/20 sec and 10/sec) of the miniature end-plate potentials (Fatt & Katz, 1952; Furshpan, 1956). Hypertonicity also often caused an increase in the size of the miniature end-plate potentials. This effect was perhaps due in part to a reduction of the fibre diameter (Katz & Thesleff, 1957). Most experiments were made at between 17 and 21° C; a few were made at 37° C.

Muscles. The muscles used were the caudal belly of the biventer cervicis, the semispinalis cervicis, the anterior and posterior parts of the latissimus dorsi and the extensor carpi radialis profundus (for the anatomy of the chick see Chamberlain, 1943). I am indebted to Professor Eleanor Zaimis for introducing me to the neck muscles, which were first described as isolated preparations by Child & Zaimis (1954) and Child (1955). Except for the extensor carpi radialis profundus, the muscles are composed of parallel bundles of fibres

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held together by several layers of closely woven connective tissue. This was more easily removed from the dorsal than the ventral surfaces of the muscles; it was, however, not possible to remove the connective tissue entirely from either part of the latissimus dorsi without causing extensive damage to the muscle. In the neck muscles, the biventer cervicis and the semispinalis cervicis, it was found (by stimulating and recording from curarized preparations with external electrodes) that the fibres do not run the length of the muscles, but are arranged in the same way as in the tenuissimus of the cat (Adrian, 1925), the average length of a fibre (about 1 cm in a 14-day-old chick) being about one third the length of the muscle. The average diameter of the fibres in the biventer cervicis varied from about 12 μ in muscles taken from chicks 1 day old to 35 μ at 6 weeks, these values being obtained by measuring isolated single fibres. An increase in diameter with age was confirmed by measuring the average diameters in photographic enlargements of slides of transverse sections kindly provided by Dr B. Mackay, of the Anatomy Department, Edinburgh. The diameters, relative to that in a 1-day chick, were 1·2, 1·6, 1·8, 2·1 and 2·5 at 1, 2, 3, 4 and 6 weeks, respectively. As would be expected on the basis of the relatively constant increment in membrane conductance underlying the miniature end-plate potential (Katz & Thesleff, 1957), larger miniature end-plate potentials could generally be recorded from muscle fibres of younger birds.

The usual difficulty associated with inserting electrodes into small fibres was encountered (cf. Katz & Thesleff, 1957; Kuffler & Vaughan Williams, 1953*a*; Burke & Ginsborg, 1956); i.e. the impalements were unstable and the resting potentials generally low and sensitive to small vertical movements of the micro-electrode. The difficulty was aggravated, especially at higher temperatures, in many cases of the biventer cervicis, the semispinalis cervicis, and the anterior part of the latissimus dorsi, by small movements within the muscles which could not be abolished and which dislodged the electrodes. The two highest resting potentials recorded were 84 mV, on the one occasion in a fibre of the biventer cervicis from which focal miniature end-plate potentials were recorded, and on the other in a fibre of the same muscle from which diffuse spontaneous activity was recorded.

RESULTS

Focal activity

'Focal' spontaneous activity, illustrated in Fig. 1, has been recorded from twenty-five fibres in three different varieties of muscle, namely, the biventer cervicis, posterior portion of the latissimus dorsi, and the extensor carpi radialis profundus. In the two latter muscles it was the only type of activity encountered and it is noteworthy that in the majority of impalements in these two muscles no spontaneous activity was seen. Presumably, therefore, it was necessary for the impalement to be made at some precise position close to a focal neuromuscular junction for this variety of activity to be seen. In the biventer cervicis focal activity was observed in a small minority of impalements, electrical silence in a larger minority and diffuse activity, to be described below, in the majority of cases. All spontaneous electrical activity was abolished by tubocurarine 5×10^{-6} (w/v). The focal activity clearly consisted of a series of miniature end-plate potentials similar to that which has been observed in twitch fibres in the frog (Fatt & Katz, 1952) and in the mammal (Boyd & Martin, 1956; Liley, 1956; Brooks, 1956). The mean amplitudes varied between 0·6 and

3·1 mV from fibre to fibre. In individual fibres the time to the peak, at room temperature, varied between 4 and 12 msec, but in any one fibre the rise time did not vary by more than a factor of 2 and there was no

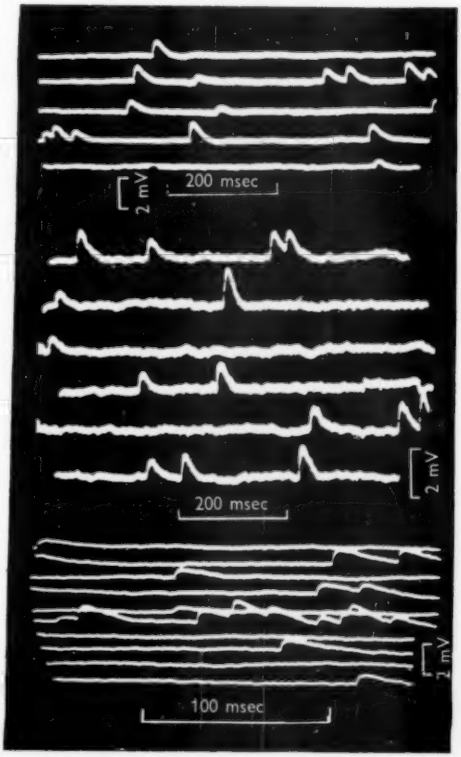

Fig. 1. Spontaneous electrical activity recorded with an intracellular electrode in three different fibres of the biventer cervicis. Temperature, 19° C. The relatively constant shape of the spontaneous potentials in each fibre indicates that they originated at a focal end-plate close to which the fibre was impaled.

obvious relation between amplitude and rise time (cf. del Castillo & Katz, 1956). The variations from fibre to fibre are no doubt largely accounted for by differences in fibre size (Katz & Thesleff, 1957) and, since electrodes were inserted at random, by differences in the proximity of the electrode to the end-plate. As is the case in the frog (Fatt & Katz, 1952) the frequency distribution of amplitude appears to approximate to a normal distribution with a coefficient of variation of about 30% (Fig. 2). The relative constancy of 'shape', and the normal frequency distribution of

amplitudes are both consistent with the view that the 'focal' spontaneous activity did indeed originate at a focal end-plate near which the electrode was inserted.

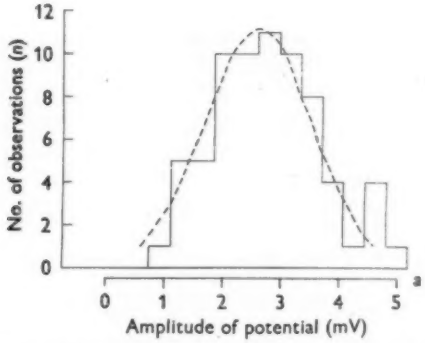

Fig. 2. Frequency distribution of the amplitudes of a series of 70 'focal' miniature end-plate potentials recorded from a single fibre of the biventer cervicis. The dotted curve is the theoretical normal distribution $n = \frac{70}{0{\cdot}93\sqrt{\pi}} \exp\left[-\frac{(a-2{\cdot}6)^2}{2\times(0{\cdot}93)^2}\right]$ for a population equal to the number of observations, with the same mean and variance as were observed.

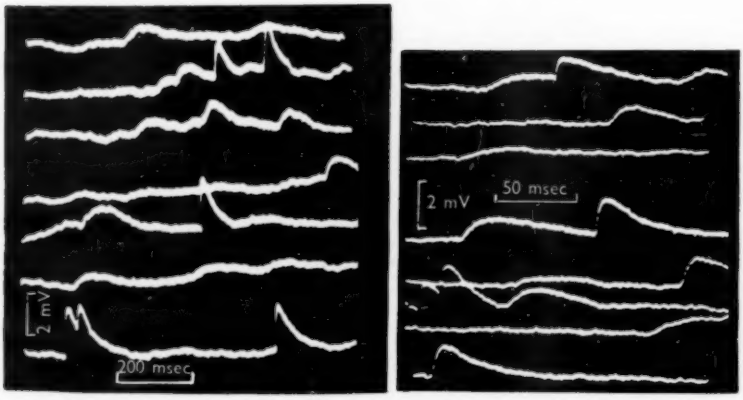

Fig. 3. 'Diffuse' spontaneous activity in a fibre of the biventer cervicis. Temperature 19° C. The variations in shape indicate that the miniature end-plate potentials originate at junctions at a variety of different distances from the point at which the fibre was impaled.

Diffuse activity

'Diffuse' spontaneous activity, illustrated in Fig. 3, has been observed in several hundred fibres of the biventer cervicis, semispinalis cervicis and the anterior part of the latissimus dorsi. It evidently consists of a series

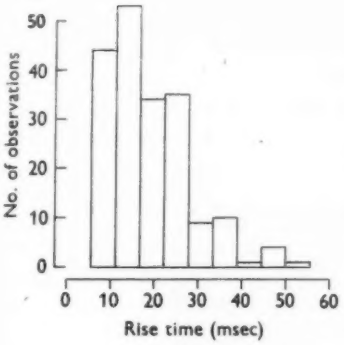

Fig. 4

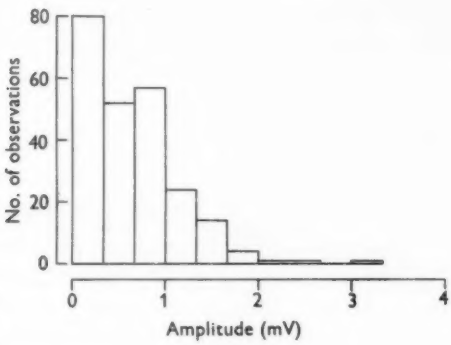

Fig. 5

Fig. 4. Frequency distribution of rise times of 191 'diffuse' miniature end-plate potentials. Same experiment as in Fig. 6.

Fig. 5. Frequency distribution of amplitudes of a series of 237 'diffuse' miniature end-plate potentials. Same experiment as in Fig. 6.

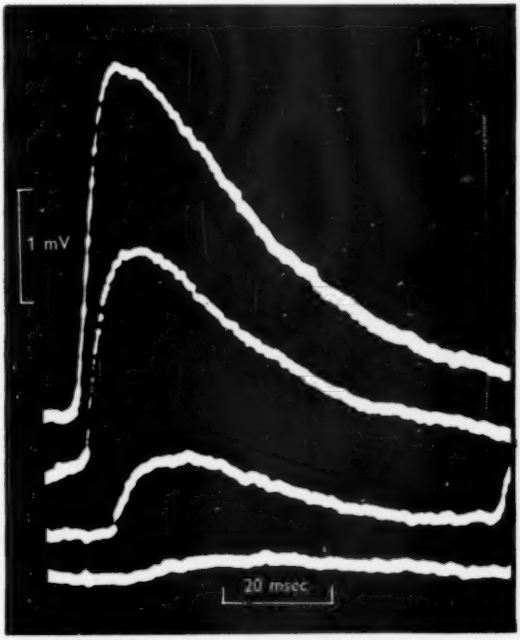

Fig. 6. A selection of miniature end-plate potentials from a single impalement of a fibre of the anterior latissimus dorsi, showing the association of longer rise times with smaller amplitudes.

of miniature end-plate potentials, but in contrast with the focal potentials there is a striking variation in rise time (Fig. 4), the frequency distribution of amplitudes is skew, smaller amplitudes occurring most frequently (Fig. 5) and small amplitudes are often associated with long times of rise (Fig. 6). These features are to be expected of miniature end-plate potentials which originate at junctions at different distances from the recording electrode, and they have in fact been observed previously by Burke (1957),

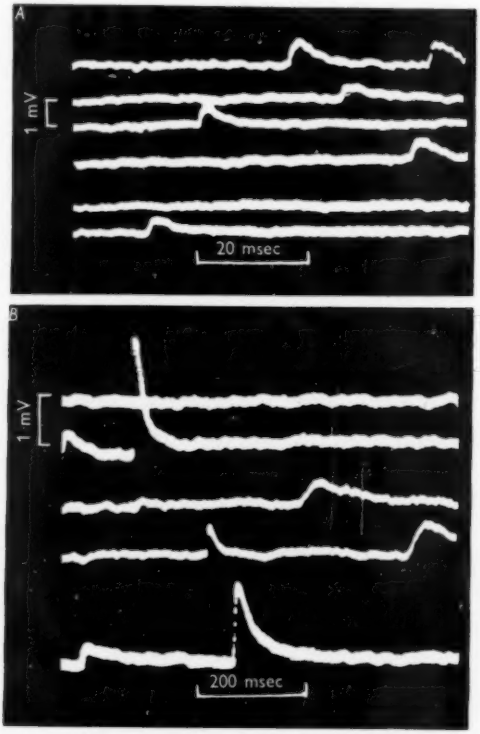

Fig. 7. Focal (*A*) and diffuse (*B*) spontaneous activity recorded from two different fibres of the same biventer cervicis muscle at 37° C.

in the spontaneous activity of 'slow' fibres of the frog which are known to have a distributed innervation (Kuffler & Vaughan Williams, 1953*a*; Burke & Ginsborg, 1956). Since most experiments were made at room temperature, which was about 20° C lower than the body temperature of the chick, it was remotely possible that the diffuse activity was related to this abnormal temperature (cf. del Castillo, 1955). The distinction, however, between focal and diffuse activity remained at 37° C (Fig. 7). Moreover, in

the anterior latissimus dorsi, diffuse activity was the only type of spontaneous activity observed and it occurred in the case of almost every impalement. This observation alone strongly suggests the existence of a variety of muscle fibre with neuromuscular junctions distributed over the length of the fibre. It also disposes of the possibility that the diffuse activity arose only from intrafusal fibres of spindles.

Simultaneous recording with two micro-electrodes

A method of estimating the density of innervation of a fibre is to insert two electrodes at known distances from one another in the same fibre and to record from them simultaneously. (Since it was not possible to follow these fibres visually, the second electrode was inserted as a current-passing electrode, a successful impalement being registered by the appearance of an electrotonic potential in the first recording channel; by actuating a micro-switch, the second electrode was then connected to the input of a second recording channel.) Results from three fibres are illustrated in Fig. 8. It may be noted that, apart from providing some estimate of the density of innervation, these results unequivocally demonstrate the existence of a distributed innervation, since the range of amplitudes of the miniature end-plate potentials recorded by either electrode was the same. Nor was this the result of the two electrodes being either side of, and equidistant from, a single source. It is, for example, apparent from Fig. 8, (*a*) that the miniature end-plate potential, α, recorded on channel *B* originated at a point relatively close to electrode *B* and too distant from electrode *A* to be recorded by it; whereas the two miniature end-plate potentials, β, originated between the two electrodes, and the miniature end-plate potential, γ, originated relatively close to electrode *A* and distant from *B*. There must therefore be at least three distinct junctional regions in the field of recording of the two electrodes. In fact, in this experiment the ratios of the amplitudes of the nineteen miniature end-plate potentials which were recorded simultaneously by the two electrodes fell into at least five separate classes. It is unlikely that any miniature end-plate potentials arising outside the length of the fibre between the two electrodes would have been seen simultaneously since the distance between them was 1·7 mm, which is equivalent to about $2{\cdot}5\,\lambda$, λ being the space constant of the fibre, and a miniature end-plate potential originating at one electrode would be attenuated by more than one-twelfth, $e^{-2{\cdot}5}$, in amplitude at the other electrode (λ was found to be about 0·7 mm in two different fibres); in this fibre it is therefore probable that the average distance between the junctional regions at which the miniature end-plate potentials arose was not more than $340\,\mu$. The majority of miniature end-plate potentials were seen only on one or other channel, presumably partly because they

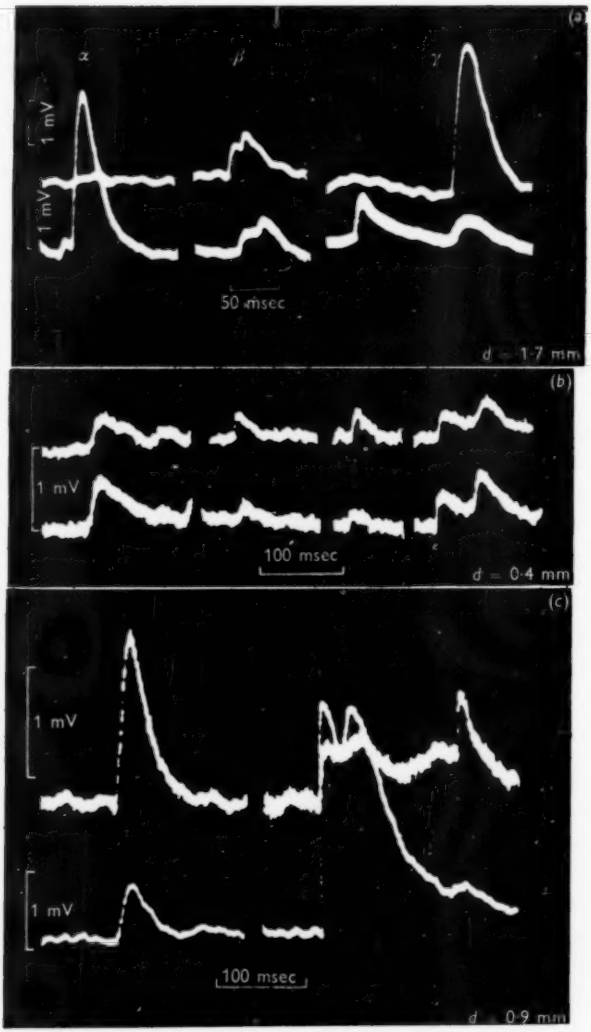

Fig. 8. Simultaneous records from two intracellular electrodes separated by different distances, *d*, in three fibres, (*a*), (*b*) and (*c*), of the anterior latissimus dorsi. The upper traces in each set of (*a*), (*b*) and (*c*) are from the one recording channel, and the lower traces from the other (see text).

arose from points outside the segment between the two electrodes and partly because only the largest of the miniature end-plate potentials which arose close to one electrode remained above the noise level at the other electrode. A similar estimate for the distance between adjacent sources of miniature end-plate potentials is suggested by Fig. 8(*b*), in which the two recording electrodes were separated by 400 μ in a fibre in which the largest miniature end-plate potentials were less than 1 mV. Only those arising from a junction close to the electrode would be recorded by either electrode above noise level. Even so, it was apparent that there were at least two separate sources of activity both within or at any rate close to the 400 μ segment included by the electrodes. In five of the fibres investigated it was clear that at least one of the two electrodes was not properly inserted; no analysis of the distribution of junctions was therefore possible. In every experiment, however, it was evident that miniature end-plate potentials arose from sources sometimes closer to one electrode and at other times closer to the other.

DISCUSSION

The results described in this paper show that certain muscles in the chick contain fibres in which neuromuscular junctions occur at many points along the fibre. The distance between adjacent junctions in these fibres is, at the most, of the same order of magnitude as the space constant of the fibre; pharmacological agents which cause depolarization at the neuromuscular junction may therefore be expected to cause a relatively uniform depolarization of the surface of a diffusely innervated fibre. Allowing that contraction is intimately related to depolarization, it seems likely that it is these fibres which are responsible for the contracture of such avian muscles as respond with contractures, which occur on the application of acetylcholine-like substances (e.g. Langley, 1905/1906; Brown & Harvey, 1938; Buttle & Zaimis, 1949; Paton & Zaimis, 1952).

The similar contractures which occur in certain amphibian muscles are known to be due to the activity of a distinct group of fibres, the slow fibres (see Kuffler & Vaughan Williams, 1953*b*), which are also known to be diffusely innervated (Kuffler & Vaughan Williams, 1953*a*; Burke & Ginsborg, 1956; Burke, 1957). It should therefore be of interest to compare other properties of the diffusely innervated amphibian and avian muscle fibres. A notable difference, which has already been briefly reported (Ginsborg, 1959), is that, unlike the amphibian slow fibres, diffusely innervated fibres in the biventer cervicis of the chick are capable of responding to adequate stimulation with propagated action potentials.

In the frog there is a striking correspondence between the distributions amongst various muscles of slow fibres and of the fibres described

histologically by Krüger as being of 'Felderstruktur' (see Krüger, 1952; Kuffler & Vaughan Williams, 1953*b*; Gray, 1958). These 'Felderstruktur' fibres have been supposed by Krüger to be responsible for contractures and in the frog they have been shown by Günther (1949) to be endowed with 'en grappe' type of nerve endings as opposed to the typical endings on twitch fibres. I am indebted to Mr A. F. Huxley for suggesting that a correspondence might also exist between diffusely innervated fibres and 'Felderstruktur' fibres in the bird; and that it might be revealed by comparing the two parts of the latissimus dorsi muscle. These two parts, the anterior and posterior, have been found by Krüger (1950, 1952), in a variety of species of bird, to be made up exclusively of 'Felderstruktur' and 'Fibrillenstruktur' fibres, respectively, the Fibrillenstruktur fibres being endowed with typical end-plates and, as in the frog, the Felderstruktur fibres having 'en grappe' nerve terminations. In addition, in one experiment, on the crow, Krüger found that the anterior latissimus dorsi responded to acetylcholine with a contracture whereas the posterior part did not. In the present experiments, as has already been noted (pp. 708 and 713) no diffuse spontaneous activity has ever been observed in the posterior latissimus dorsi and no focal activity has been observed in the anterior latissimus dorsi. Whilst these results cannot be regarded as conclusive, since only a small proportion of the fibres in any muscle have been impaled and furthermore the chick was not among the birds investigated by Krüger, they are, nevertheless, highly suggestive of an identification of the diffusely innervated fibres with those described by Krüger as 'Felderstruktur' fibres.

SUMMARY

1. The spontaneous activity at the neuromuscular junction in muscle fibres of the chick has been investigated with intracellular electrodes.

2. The activity provides evidence of two types of fibre, one with a focal end-plate and the other with junctions distributed at many points along the fibre.

3. The distribution of the diffusely innervated fibres amongst the various muscles investigated, suggests that they may correspond to Krüger's 'Felderstruktur' fibres and that they may be responsible for the contractures obtained in avian muscle on the application of acetylcholine-like substances.

I am deeply indebted to Professor W. L. M. Perry for advice, encouragement and hospitality, to Professor D. Whitteridge for the use of many facilities and to Messrs W. T. S. Austin and N. E. Condon for their unfailing help.

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PROCEEDINGS OF THE PHYSIOLOGICAL SOCIETY

CHARING CROSS HOSPITAL MEDICAL SCHOOL MEETING

11–12 *December* 1959

Cytochemical and radio-isotope techniques applied to the study of the hypothalamus and neurohypophysis. By BARBARA C. KING and J. C. SLOPER. *Department of Morbid Anatomy, Charing Cross Hospital Medical School, London, W.C. 2*

Wireless telestimulation of the cat's motor cortex with constant stimuli. By S. BONAZZOLA and T. GUALTIEROTTI*. *Physiological Laboratory of the University, Milano, Italy*

Wireless stimulation of the cat's cortical motor centres at a distance of up to 50 yards has been achieved by using a receiver-stimulator developed in order (*a*) to be small enough to be chronically implanted under the animal's skin, and (*b*) to apply stimuli of a constant amplitude and duration whatever the position and the movements of the animal.

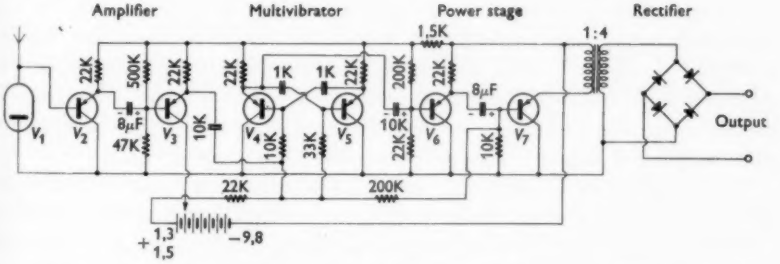

Fig. 1. V_1 = OA85; V_2, V_3, V_4, V_5, V_6 = OC72; multivibrator = 2–7000 c/sec.

The all-transistor device (Fig. 1) consists of a receiving antenna, a double amplifier, a blocked multivibrator and a power stage with an output insulating transformer. The receiver-stimulator is powered by seven miniature mercury batteries in series, 1·45 V each. This gives an all-or-none stimulus of pulses of 1–2 msec duration (or more at will) at a strength of up to 100 mA. These pulses are conducted to the required

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nervous centres through implanted wire electrodes. The apparatus is triggered whatever the position of the animal, owing to the high gain of the amplifier stage, at a distance of up to 50 yards even through concrete or wooden walls.

A 30 cm wave-length transmitter with a parabolic antenna is used for triggering the stimulator at any required frequency. The stimulation of discrete centres of the motor cortex has been possible in cats for approximately 1 month after the implantation of the device before the cells were exhausted. If a longer duration is wanted, the mercury batteries may be kept disconnected and connexion made through a couple of wires contained in a polythene tube protruding from the skin without any inconvenience to the animal.

Various movements, according to the position of the stimulating electrodes in the cortex, have been induced and the behaviour of the animal observed. The conclusion has been drawn that the cat is apparently unaware of, and not troubled by, the cortical stimulation and the following movements.

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A cine technique for measurement of eye movements in a dynamic environment. By W. T. Free and G. M. Jones*. *R.A.F. Institute of Aviation Medicine, Farnborough, Hants*

The apparatus comprises a standard closely fitting flying helmet, having a forward facing cine camera running at 16, 32 or 64 frames/sec, rigidly mounted on one side, with a 2 in., F/1·9 lens which carries a periscope so arranged that the camera takes a close-up picture of one eye. The motor is connected to the camera by a flex-drive and is mounted on the opposite side for inertial balance. The periscope has front surfaced mirrors, carries a 12 W half-silvered bulb for illumination of the eye and contains an annular photo-cell for exposure control, the current from which is amplified approx. 10 times by a transistorized circuit. A hood is available for covering the periscope and eye, mainly to reduce corneal reflexions to a single spot of light. The whole system can be fixed relative to the skull with a dental bite. The apparatus is controlled from a small box strapped to the knee, by means of which mains voltage, bulb voltage and amplified current from the photo-cell can be measured, and bulb current can be adjusted through a power transistor control circuit. A self-contained Cd-Ni battery pack supplies 28 and 18 V at 1 amp each for 12 min.

Eye movement in the 'horizontal' and 'vertical' planes can be measured

* External staff, Medical Research Council.

to an accuracy of approx. ± 1° within 25° of a central datum by measuring displacement of the pupil image on the film. Rotational movement about the visual axis can be measured with a similar accuracy by superimposing a projected image of the iris upon a disk, free to rotate about its

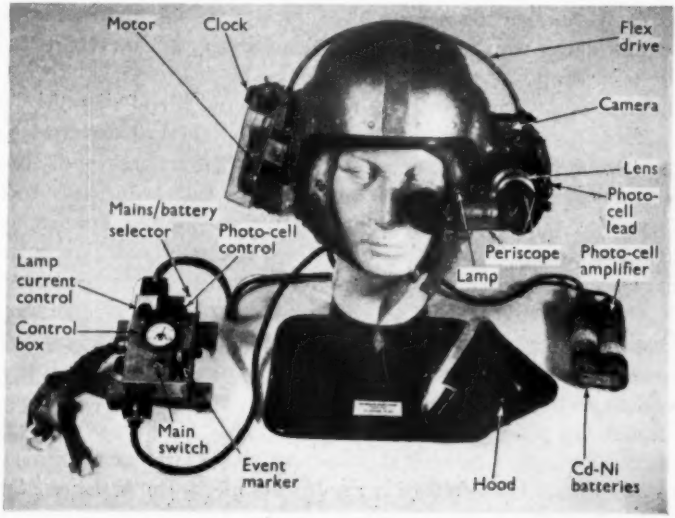

Fig. 1. Apparatus for measurement of eye movements.

axis and having characteristic radially disposed marks of the particular iris sketched upon it. The disk is mounted upon a support, graduated in degrees, which is constrained to move in the plane of the image without itself incurring rotation.

The effects of intense sound on the cochlea of the guinea-pig. By W. BURNS and R. P. GANNON. *Physiology Department, Charing Cross Hospital Medical School, London, W.C. 2*

Observations on the rhesus monkey *in utero*. By G. S. DAWES, H. N. JACOBSON, JOAN C. MOTT and HEATHER J. SHELLEY. *The National Institute for Neurological Diseases and Blindness, Field Station of Perinatal Physiology, San Juan, Puerto Rico, and the Nuffield Institute for Medical Research, University of Oxford*

Observations have been made on twelve foetal monkeys *in utero* under pentobarbitone anaesthesia, ranging in gestation age from 115 to 158 days (term is about 168 days). A leg was delivered through a small uterine

incision and a catheter was inserted into the femoral artery for recording blood pressure and to obtain blood samples. In five foetal monkeys an arm was also delivered through a separate incision and the brachial artery was catheterized so that samples could be withdrawn from tributaries of the ascending and the descending aorta simultaneously. In experiments on the three youngest monkeys (126 days gestation or less) a uterine incision was made over the neck of the foetus and a catheter was introduced into the left carotid artery.

The mean O_2 saturation of the femoral arterial blood was 58% (range 51–63). Simultaneous samples taken from a brachial artery were, on the average, 9% higher; values as high as 77 and 79% were observed. The blood lactate concentration was of the same order as that seen in six monkeys 8–12 days after birth (7–17 mg/100 ml.). In three foetal monkeys, femoral arterial blood samples withdrawn at the peak of a uterine contraction (producing a rise of intra-uterine pressure of up to 35 mm Hg) contained less O_2 (by 1·5–9% saturation) than did samples withdrawn during a quiescent period between contractions. On delivery of the foetus the uterus contracted strongly and the foetal arterial O_2 saturation fell to low values, comparable with those commonly observed in human cord blood samples, even though the placenta was not yet separated. It is therefore suggested that cord blood samples, taken after delivery, may give a misleading impression of the conditions of intra-uterine life in the primate. And it is interesting that the same difference between the oxygen content of the blood in the ascending and the descending aorta exists as has been shown previously in ungulates.

This work was carried out with the aid of a grant from the United Cerebral Palsy Research and Educational Foundation.

The action of 2-4-DNP on rat auricular action potential duration and intracellular levels of Na^+ and K^+. By J. F. Lamb. *Department of Veterinary Physiology, University of Edinburgh*

Weidmann (1956) showed that if the external potassium concentration $[K]_O$ was increased in the Ringer's solution perfusing turtle hearts the action potential shortened, and Délèze (1959) showed that a decrease in the external sodium concentration $[Na]_O$ also shortened the action potential. Weidmann was able to show that the shortening of the action potential was independent of the drop in resting potential accompanying the shortening, but it has not been established if the changes in the duration of the action potential are due to the alteration in the $[K]_O$ or $[Na]_O$ *per se*, or whether they are related to the concomitant changes in the equilibrium potentials of the ions (E_K or E_{Na}). The present experiments were designed

to test this point, by altering the intracellular values of K and Na in rat auricles and observing the effect on action potential duration. Use was made of 2-4-dinitrophenol (DNP), which was shown in preliminary experiments to affect the internal concentrations of K^+ and Na^+, $[K]_I$ and $[Na]_I$, and which is known to shorten the action potential duration (Macfarlane, 1956; Webb & Hollander, 1956).

Treatment	No. of expts.	$[Na]_I$ (m-mole/kg fibre water)	$[K]_I$ (m-mole/kg fibre water)	Resting potential (mV)	Overshoot (mV)	Action potential duration (msec)
T 1 min	8	40·0 (8·6)*	159 (7·1)	80 (1·4)	28 (1·1)	55 (4·7)
T 30 min	4	46·4 (2·3)	132 (2·5)	81 (0·8)	27 (2·8)	45 (3·8)
DNP 15 min	11	46·0 (7·9)	113 (6·7)	71 (3·0)	3 (3·6)	6 (1·2)
DNP 15 min +T 15 min	7	53·1 (6·8)	132 (5·7)	78 (2·5)	16 (2·4)	35 (3·4)

* Standard error of the mean.

T is tyrode, DNP is 2-4-dinitrophenol, and the measurement of the duration is made at 90% repolarization.

Left auricles of rat heart were perfused at 35° C with Tyrode solution (157 m-mole Na and 2·7 m-mole K per litre) or Tyrode solution containing 13 μmole DNP and equilibrated with 95% O_2 and 5% CO_2, and driven at 60/minute. Action potentials were recorded by intracellular electrodes and at the end of each experiment the hearts were dried, wet-ashed and analysed for Na and K content (Goodford, 1959). Some hearts were perfused with choline chloride solution at 6° C for varying periods to determine the extracellular space (Krnjević, 1955; Dainty & Krnjević, 1955). The value obtained was 25 ml./100 g wet weight.

Table 1 shows that DNP causes a large fall in $[K]_I$ but only a small increase in $[Na]_I$. Recovery from DNP is associated with an increase of $[K]_I$ but without any reduction of $[Na]_I$. A comparison between the groups of hearts with the same $[K]_I$ or $[Na]_I$ values shows that an increase of $[Na]_I$ or a decrease in $[K]_I$ both shorten the action potential. Thus alteration of E_K or E_{Na} either by changing the internal or the external concentrations of the ions shortens the action potential. The action potential duration, however, appears to be more sensitive to changes in $[K]_I$ than to the corresponding change in $[K]_0$ required to alter the E_K by the same amount.

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incision and a catheter was inserted into the femoral artery for recording blood pressure and to obtain blood samples. In five foetal monkeys an arm was also delivered through a separate incision and the brachial artery was catheterized so that samples could be withdrawn from tributaries of the ascending and the descending aorta simultaneously. In experiments on the three youngest monkeys (126 days gestation or less) a uterine incision was made over the neck of the foetus and a catheter was introduced into the left carotid artery.

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Biological activity of pure bradykinin—a plasma kinin from ox blood. By D. F. Elliott, E. W. Horton and G. P. Lewis. *National Institute for Medical Research, London, N.W. 7*

The plasma kinin bradykinin has been isolated and was found to contain the following amino acid residues: arginine, phenylalanine, proline, serine, glycine, in the proportion 2:2:2:1:1 (Elliott, Lewis & Horton, 1959).

In the present investigation the biological activity of the pure polypeptide has been compared with a crude preparation similar to those used in most other investigations. The two were compared on the following isolated smooth-muscle preparations, the guinea-pig ileum, rat uterus, rat duodenum and rabbit duodenum. In addition, comparisons were made for vasodilatation in the skin of cat hind limb, for depressor activity by intravenous injection into cats and rats, and for effect on capillary permeability by intradermal injection into the skin of guinea-pigs and rabbits. It was found that pure bradykinin was active on all these preparations and within the errors of biological variation the increase in activity from the crude material was proportional in all tests.

All these activities are therefore inherent properties of the pure polypeptide which is probably mainly responsible for the biological activity of the crude mixture of polypeptides. However, the observations do not exclude the possibility of the presence of other active peptides differing only quantitatively from bradykinin.

The active concentrations of the pure polypeptide varied between 0·2 ng/ml. required to contract the rat uterus and 400 ng/kg to cause a depressor response in cats. On a molar basis, bradykinin was approximately as active as acetylcholine as a vasodilator and about 10 times more active than histamine in increasing capillary permeability.

Bradykinin therefore ranks among the most active of biological substances, and may be responsible for all the actions of the group of polypeptides which have been classified under the name of plasma kinin.

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Dinitrophenol and potassium exchanges in rabbit auricles. By M. Weatherall. *Department of Pharmacology, London Hospital Medical College, London, E. 1*

The effects of 2:4-dinitrophenol (10^{-4} M) on potassium and ^{42}K movements in rabbit auricles have been studied under conditions described by Rayner & Weatherall (1959). This concentration of dinitrophenol arrested

spontaneous beating within 5 min of application. Net loss of potassium occurred, but was not more than 20 % until after 3 or more hours. The loss was much smaller than expected from the reported decline in resting membrane potential under rather similar conditions (de Mello, 1959). The entry of ^{42}K was not significantly altered in the first hour of uptake, whether auricles had been exposed to dinitrophenol for an hour before immersion in radioactive medium or only during the period of ^{42}K uptake. Efflux of ^{42}K from tissues which had been immersed in tracer for 2–6 hr was

TABLE 1

Treatment	^{42}K efflux rate constant (min^{-1}; mean ± S.E.) Left auricles (quiescent)	Right auricles (beating until DNP treated
No. of auricles ...	4	3
Control before DNP	0·0164 ± 0·0004	0·0277 ± 0·0019
DNP 10^{-4} M	0·0229 ± 0·0014	0·0289 ± 0·0021
Control after DNP	0·0148 ± 0·0005	0·0209 ± 0·0024

accelerated (Table 1). There was a steady net gain in sodium during immersion in medium containing dinitrophenol. The results suggest that dinitrophenol increased the permeability of the cell walls to potassium, but they could also result from a failure to concentrate potassium in a bound fraction (Weatherall, 1959) if this fraction was accessible only through the main cellular fraction.

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Haemoglobin concentration and total haemoglobin in normal kittens. By SATYAVATHI GURUBATHAM and PATRICIA P. SCOTT. *Department of Physiology, Royal Free Hospital School of Medicine, London, W.C.* 1

Working with J. P. Greaves we measured the Hb concentration and packed cell volume in 158 venous blood samples from healthy kittens, of which only the youngest were anaesthetized. Hb concentration was 17·12 ± 0·43 g/100 ml. blood (estimated with an M.R.C. grey-wedge photometer) at birth, but fell rapidly during the first week of life to between 6 and 7 g/100 ml. blood at 21–35 days, after which it rose, reaching 10·96 ± 0·35 g at 10 weeks of age. Packed cell volume followed a similar course, so that the mean corpuscular Hb remained fairly constant, averaging 36·7 % over all observations.

We have now determined total Hb, by the viviperfusion method described by Hallgren (1954) for rats, on groups of six to eight kittens, ranging from new-born to 12 weeks of age, litter-mates being distributed throughout the groups. In the first 18 hr Hb was $1{\cdot}07 \pm 0{\cdot}09$ g/100 g body weight. A marked individual variation in total Hb explained the wide variation in Hb concentration observed, but these variations were not related to birth weight or litter size. In spite of progressive fall in Hb concentration and in Hb/unit body weight (minimal level of $0{\cdot}43 \pm 0{\cdot}034$ g/100 g body weight) the total Hb remained constant during the post-natal period until the third week when a just significant rise ($P = < 0{\cdot}05$) was first observed; the very low Hb concentration noted at this stage appeared to provide a stimulus for erythropoiesis. At 5 weeks the total Hb was more than double the value at birth. This increase prevented any further fall in concentration but was only just sufficient to compensate for the growth of other tissues in this period. During the next period, when solid food was introduced into the diet, the rate of formation of Hb increased rapidly. By the 8th week the proportion of Hb to other tissues reached a steady state at $0{\cdot}56 \pm 0{\cdot}018$ g/100 g body weight which was maintained until the kittens were 6 months old.

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The survival of new-born rats in nitrogen. By Anne Stafford and Josephine A. C. Weatherall. *Nuffield Institute of Medical Research, University of Oxford*

New-born rats exposed to nitrogen survived for (i.e. took their last gasp at) about 28 min at an environmental temperature of 36° C. Blood and tissue carbohydrate and lactate were measured by the methods described by Dawes, Mott & Shelley (1959) in rats which had been exposed to nitrogen for varying periods of time. There was a fall in heart and liver carbohydrate and a rise in blood glucose, but little change in the carbohydrate content of skeletal muscle, lung or skin; the fall in liver and heart carbohydrate accounted for about 65 and 10% respectively of the lactic acid production in the whole rat.

In spite of this utilization of liver carbohydrate, the survival time of new-born rats in nitrogen was more closely related to the initial heart carbohydrate than to the liver carbohydrate or blood glucose concentrations. For instance:

(1) When new-born rats were deprived of food before exposure to nitrogen, their survival time decreased in proportion to the decrease in heart carbohydrate; there was a much greater fall in liver carbohydrate.

(2) When rats were exposed to nitrogen for 23–25 min and replaced in air the heart carbohydrate returned to the initial level within 3 hr, but the liver carbohydrate remained low. When litter-mates, which had been treated similarly, were now subjected to a second period of anoxia their survival time in nitrogen was directly related to the heart carbohydrate and not to the liver carbohydrate concentration.

(3) With increasing age up to 16 days from birth, both the cardiac carbohydrate and the survival time in nitrogen decreased gradually. In contrast, liver carbohydrate fell rapidly to a minimum after the first day, at which time the survival time was still high, and later increased as the survival time fell.

Intraperitoneal injection of glucose before exposure to nitrogen did not prolong the survival time of normal new-born rats, which died with a high blood glucose. Glucose did considerably increase the survival time of rats whose blood sugar and heart carbohydrate had been reduced to an abnormally low level by a previous period of anoxia, from about 17 to 28 min.

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The sensitivity of conscious human subjects to added external elastic impedance to breathing. By E. J. M. Campbell, S. Freedman, P. S. Smith and M. E. Taylor. *Medical Unit, Middlesex Hospital, London, W.* 1

Healthy adult males, breathing air through a circuit of negligible resistance, were made to inspire at intervals from a series of closed metal drums containing air initially at atmospheric pressure. These provided an added external elastic impedance in inverse proportion to their volumes. The elastances used were 4·9, 3·45, 2·6, 2·0 and 1·4 cm H_2O/l., and each of these was presented, unknown to the subject, six times, in a randomized order. The subject was required to signal detection of the impedance. At each presentation of an impedance, five breaths were allowed for detection. Five points were scored for a detection in the first of the five allowed breaths, and nil if the impedance remained undetected. Percentage detection was plotted against the value of the added elastance, and also against peak inspiratory mouth pressure. From the graphs so obtained, the values of added elastance and of peak inspiratory pressure which were detected on 50% of occasions ('50% detection levels') were calculated. Six subjects were each studied twice, at intervals from 2–5 weeks.

The mean 50% detection level of elastance was 2·47 cm H_2O/l.

(S.D. 0·408; $n = 12$). There was no significant difference between the average values for the first and second studies. Similar, or greater, values of increased elastance (decreased compliance) of the lungs occur in disease.

The mean 50 % detection level for peak inspiratory pressure at the mouth was 1·78 cm H_2O (S.D. 0·576; $n = 12$). There was a significant average decrease (0·5 cm H_2O; S.D. 0·108) between the first and the second study. This was associated with a corresponding reduction in average tidal volume.

When the tidal volumes were increased by the addition of a dead space of 800 ml. the percentage detection rate of added elastance was increased in most, but not all, subjects.

The tidal volume was not significantly changed in those breaths during which added impedance was detected. The results are most easily explained by the presence of a mechanism which, during the course of each breath, correlates changes of trans-thoracic pressure with changes of thoracic volume (or, changes of muscle tension with changes of muscle length).

The thickness of the arterial wall. By D. H. BERGEL. *Department of Physiology, St Bartholomew's Hospital Medical College, London, E.C.* 1

The ratio of the wall thickness to the radius of an artery appears as one of the factors determining pulse wave velocity in the Moens-Korteweg equation. It has been shown repeatedly that this velocity increases towards the periphery, and it has been thought that this is largely due to an increase in relative wall thickness in the smaller vessels.

When an artery is removed from the body it shortens or retracts; this will alter the wall thickness, as will the collapse following removal of distending pressure. It follows that when measurements are made on isolated arteries both factors must be considered. Hürthle (1920) appears to be the only worker whose results are acceptable on this account. He fixed arteries *in situ* at a pressure of 130 cm H_2O and made measurements on cut sections, and reported a constant value for the ratio $h/R_0 \times 100$ (h = wall thickness, R_0 = external radius) of 15 for the whole arterial system, with the exception of the aortic root where it was 16–20. However, his results derive almost entirely from one dog, for the method is difficult and tedious. A different approach has been used here, which is based on the fact that the arterial wall does not change in volume when stretched (Lawton, 1954); thus the wall thickness of a cylindrical vessel may be calculated from its external dimensions.

Using dogs' arteries the retraction on excision was determined and the volume measured by Archimedes's principle. The apparatus previously

described (Bergel, 1958) was used in which the vessel was extended to its *in vivo* length and the radius measured photo-electrically at any internal pressure. The calculated relative wall thicknesses at 100 mm Hg pressure for the four types of vessel studied were, thoracic aorta $10{\cdot}5 \pm 0{\cdot}46$ (S.E.M.) ($n = 15$), abdominal aorta $10{\cdot}5 \pm 0{\cdot}61$ ($n = 9$), femoral artery $11{\cdot}5 \pm 0{\cdot}56$ ($n = 11$) and carotid artery $13{\cdot}2 \pm 0{\cdot}75$ ($n = 13$). A few specimens were later fixed under pressure and sectioned. Measurements on these confirmed the calculated values.

It can be seen that the arterial wall is rather thinner than was found by Hürthle (1920), and that there is a slight increase in wall thickness in the smaller vessels, but this would only account for some 10 % increase in the pulse wave velocity from the aorta to the carotid. Shortening varied from 31·9 to 42·0 % for different types of artery and is large enough to demand consideration in any study of the properties of excised vessels.

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Observations on 24-hour oxygen consumption and CO_2 excretion during carbohydrate feeding. By B. Furnass*. *The Medical Unit, Middlesex Hospital, London, W.* 1

Kekwick & Pawan (1957) observed different rates of weight loss in obese subjects fed on 1000-calorie high fat and high carbohydrate diets. Estimates of energy exchange on a time–motion basis, with the integrating motor pneumotachograph devised by Fletcher & Wolff (1954), detected no significant difference in calorie expenditure between the two diets based on the classical combustive theory of energy expenditure. These studies revealed, however, an estimated 24 hr respiratory quotient during carbohydrate feeding which was lower than that expected on the combustive theory, a finding in accord with the observation of Hagedorn (1928) of low basal respiratory quotients in obese patients fed on high carbohydrate diets.

In order to pursue these observations a respiratory helmet was devised for the accurate measurement of total pulmonary 24 hr oxygen consumption and CO_2 excretion (Furnass, 1959). Subjects were fed for a preliminary 4 days on a 2500-calorie 80 % carbohydrate diet in the hope of saturating their body glycogen stores. On the day of study 270 g (*ca.* 1000 calories) of anhydrous dextrose dissolved in lemon juice was given by tube

* At present holding a grant from the Medical Research Council.

through the respiratory helmet, the last portion being administered 14 hr before the end of the experiment. As a control 1000 calories of cream were similarly given.

Assuming total assimilation and non-storage of administered dextrose the findings in several subjects indicated a 24 hr non-protein respiratory quotient lower than that expected on the classical theory of indirect calorimetry, whereas fat feeding resulted in a quotient in the region of 0·7 in accordance with the theory. When computations were made in terms of element balance, it appears that up to 50 g per day of oxygen consumed may not have been used for combustion in the accepted sense of the word.

These observations are compatible with the hypothesis that unexpectedly low respiratory quotients may represent the combination of some absorbed oxygen with hydrogen atoms in depot fat thereby resulting in desaturation of the fat with change in its physical properties. In effect this would lead to a conservation of body carbon and body heat relative to each unit of oxygen absorbed.

It is suggested that such a mechanism could reflect a process of adaptation to the external environment, a possibility that cannot be detected by applying the commonly accepted concepts of indirect calorimetry.

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Cutaneous vasomotor nerves in the human ear and forehead.

By R. H. Fox, R. Goldsmith and D. J. Kidd. *Division of Human Physiology, National Institute for Medical Research, London, N.W. 3*

Experiments were performed to determine the type of vasomotor control in the pinna of the ear and the forehead—two areas of the skin of the head which show a marked contrast in sweat rates. The subjects were seven fit male adults. They were seated in a stirred and temperature-controlled water-bath and the blood flow through one forearm was measured by venous-occlusion plethysmography with the method described by Edholm, Fox & Macpherson (1956).

Two specially designed surface calorimeters were developed to detect changes in the rate of blood flow through the skin of the ear and forehead. The calorimeters were placed symmetrically on the two sides of the head, either near the middle of the forehead or on the upper part of the medial surfaces of the pinnae. The effects of blocking the cutaneous nerve supply

to one ear or half of the forehead on the vasodilatation and vasoconstriction of body heating and cooling were then observed. In separate experiments sweat rates from these two areas were measured.

During indirect body heating there is a large vasodilatation in the pinna of the ear which coincides approximately with the vasodilatation in the forearm skin. The vasomotor control of the ear is similar to that of the hand, being principally mediated by a vasoconstrictor release mechanism. In the forehead the results indicate predominantly an active vasodilator mechanism, as is found in the skin of the forearm, with also probably a small component of vasoconstrictor release. The association of a vasoconstrictor release mechanism in the skin of the ear, where there is little sweating, and of an active vasodilator mechanism in the skin of the forehead, where there is profuse sweating, is in agreement with the hypothesis that bradykinin-forming enzyme released from the sweat glands may be responsible for the active vasodilatation in human skin (Fox & Hilton, 1958).

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Auditory cortex of the seal (*Phoca vitulina*). By Ann M. Alderson, E. Diamantopoulos and C. B. B. Downman. *Royal Free Hospital School of Medicine, London, W.C.* 1

The rapid contractile properties of isolated large mammalian arteries. By C. J. Dickinson. *The Middlesex Hospital Medical School, London, W.* 1

Noradrenaline as a possible regulator of heat production in the new-born kitten. By R. E. Moore and Mary C. Underwood. *Department of Physiology, Royal Free Hospital School of Medicine, London, W.C.* 1

It has been shown (Moore & Underwood, 1959) that the oxygen consumption of the new-born kitten can be markedly reduced by hexamethonium, the effect declining with age over the first few weeks after birth. The suggestion was made that the response might be due to removal of an existing chemical stimulus.

A preliminary test of this hypothesis has been made on unanaesthetized kittens aged 0–37 days by subcutaneous injection of adrenaline (Ad) and noradrenaline (NAd) using oxygen consumption and body temperatures as indicators of response. Although the precise conditions have not yet been

defined, it is evident that NAd can produce a powerful and prolonged stimulation of heat production; Ad being relatively inactive. In no instance out of eight experiments did Ad in doses of 100–1000 μg/kg cause more than a 20 % increase of oxygen consumption and that short-lived, whilst in similar doses NAd in eight instances produced increases of 30–100 % lasting an hour or more.

The NAd stimulus was not always effective even in high doses, but whereas saline control or Ad injections caused no more than small and transitory disturbances, NAd on most occasions produced quite a dramatic increase in metabolism. Favourable experimental conditions for the response were a high environmental temperature (35° C) and the period of post hexamethonium depression at a lower temperature (30° C); in the latter case, however, only before the depression had become too severe.

In one experiment with an indwelling intravenous catheter in a 150 g $2\frac{1}{2}$-day-old kitten, 20 μg NA infused over 20 min reversed the fall in oxygen consumption initiated by hexamethonium with a latency of only a matter of 1–3 min.

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The influence of progesterone on placental development in the rat.

By J. F. D. Frazer, A. St G. Huggett, Muriel Jones, J. Lee and J. J. Pritchard. *Department of Physiology, St Mary's Hospital Medical School, London, W.* 2

PROCEEDINGS OF
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A model circulation with variable stroke-volume, built from simple components. By J. A. C. KNOX. *Department of Physiology, Queen Elizabeth College, London, W.8*

Collagen in coelenterates. By G. CHAPMAN. *Department of Physiology, Queen Elizabeth College, London, W.8*

The rapid action of curare on the perfused rat diaphragm preparation. By B. C. WHALER. *Department of Physiology, Queen Elizabeth College, London, W.8*

Some properties of the perfused rat diaphragm. By B. C. WHALER. *Department of Physiology, Queen Elizabeth College, London, W.8*

The rat diaphragm preparation (Bülbring, 1946) shows a fall in twitch tension and increased resting tension when stimulated at rates much greater than 12/min, which is probably due in some part to deficient oxygenation of the central muscle layers (Creese, Scholes & Whalen, 1958; Creese, Hashish & Scholes, 1958). The use of the perfused diaphragm offers the possibility of reducing these effects.

Hemidiaphragms, prepared for retrograde venous perfusion by a method similar to those given by Burgen, Dickens & Zatman (1949) and Brownlee & Straughan (1957) have been used and their responses to stimulation at fast (50/sec, medium (5/sec) and slow (3–12/min) rates tested. Indirect stimulation was used in all experiments. They were set up, in Krebs's bicarbonate saline, in 50 ml. organ baths so that they could either be perfused or treated as ordinary non-perfused preparations. Each one could therefore serve as its own control. The efficiency of perfusion was determined in the following way. Perfusion was stopped and tubocurarine

2–4 μg/ml. added to the bath fluid, to produce complete neuromuscular block. In satisfactory preparations perfusion with curare-free fluid produced a rapid and fairly complete reversal of the block even though curare was still present in the bath fluid.

Perfusion considerably reduced the fall in twitch tension which the ordinary preparation shows after indirect stimulation at 300/min for 15 min. A full tetanus was better sustained by perfused preparations. These were able to maintain a tension 30–60 % greater than that given under control conditions, although the maximum initial tension developed was not very different. After a tetanus, the response of the perfused muscle to slow stimulation showed a better recovery and a smaller reduction in amplitude compared with the pre-tetanic twitch.

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The secretion of acid by *Nepenthes*. By Shelagh M. Morrissey.
Department of Physiology, Queen Elizabeth College, London, W. 8

Some interesting analogies with the mammalian stomach are provided by the pitcher plants. For example, the tropical insectivorous plants of the genus *Nepenthes* are characterized by pitchers secreting a juice having proteolytic activity. These pitchers have glandular and non-glandular regions, the juice being secreted in the basal part which is lined with glands.

When the pitcher opens the pH of the secretion falls, and this fall in pH is probably due to the secretion of HCl. At this stage Gunzberg's test is positive. When histamine is injected into the pitcher (mean concentration 1 μg/ml. of juice, range 114 to 0·01 μg/ml.) a fall in pH can be induced. Because the pitcher will secrete acid in response to histamine it was decided to look for a similar compound in the pitcher tissue.

Extracts from glandular and non-glandular tissue were obtained by using N/3-HCl, trichloroacetic acid, or acetone as extractants. The majority of the extractions were made with N/3-HCl. When these extracts were neutralized before assay, a precipitate appeared which contained almost all the active substances. Assay of the extracts on the dorsal muscle of the leech showed the presence of an acetylcholine-like compound. This activity was partly abolished by blood esterases.

On atropinized guinea-pig's ileum the contractions obtained with glandular extract were abolished by mepyramine (mean dose of 0·1 μg mepyramine in a 20 ml. bath). This would appear to indicate the presence of a histamine-like substance. Preliminary results from chromatographic analysis of glandular extracts indicate that a compound is present having R_f values similar to those of histamine.

Assay of non-glandular extracts showed that in all cases the amount of histamine-like substance present was much less than in glandular extracts.

The thiamine-sparing activity of sorbitol and other polyhydric alcohols. By T. B. MORGAN and J. YUDKIN. *Department of Nutrition, Queen Elizabeth College, London, W.* 8

We have shown that rats and mice are able to survive on a thiamine-deficient diet if this contains sorbitol (Morgan & Yudkin, 1957). Of several other polyhydric alcohols and carbohydrates studied, only sorbose was found to replace sorbitol in this respect (Hedley & Yudkin, 1959). We have now extended this investigation and find that inositol, arabitol and pentaerythritol also have the thiamine-sparing activity of sorbitol and sorbose. With thiamine-deficient diets containing no carbohydrate, and 5 % of each of these substances, rats continued to grow and thrive throughout the 3 months of the experiments.

These substances seem to produce their sparing effect by inducing synthesis of the thiamine in the alimentary canal. However, the thiamine is not available to the animals by absorption immediately after synthesis, but only after excretion in the faeces and subsequent coprophagy (Morgan & Yudkin, 1959). Thus, rats fed a thiamine-free diet with sorbitol will begin to lose weight when prevented from having access to their faeces. The specificity of the effect was confirmed by measuring the heart rate of the rats fed a thiamine-free diet with sorbitol. Prevention of coprophagy led to the typical bradycardia of thiamine deficiency. Feeding the faeces to such an animal led to an increase in the heart rate to normal values, in the same way as giving a small dose of thiamine.

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The rate of uptake of carbon monoxide and oxygen by reduced human and sheep erythrocytes. By J. A. SIRS. *Department of Colloid Science, University of Cambridge*

A stopped-flow method based on the principles of Roughton (1934) and Chance (1940) has been developed for measurements on intact red cells. A linear relationship was found between the rate of uptake and concentration of dissolved gas, and the rate is independent of cell concentration, in agreement with the theoretical model proposed by Roughton (1932) and Nicolson & Roughton (1951).

Temperature variation. An investigation has been made of the effect, on the rate of uptake of CO and O_2 by reduced sheep erythrocytes, of temperature over the range 5–37° C. The diffusion through the membrane appears consistent with a mechanism of bulk transport, rather than by pores, and energies of activation for the process are calculated as 4350 cal/mole for oxygen and 5860 cal/mole for carbon monoxide, within an accuracy of $\pm 20\,\%$.

Ionic strength and pH effects. Measurements have been made of the effect of variation of the ionic strength on the rate of uptake of CO and O_2 by reduced sheep and human erythrocytes. Cyclic variations of the rate of uptake with decreasing and increasing ionic strength are observed and a non-linear relationship of rate with gas concentration occurs in non-isotonic media. By addition of an equivalent molar quantity of sucrose similar changes can be produced. The predominant factor is thus associated with volume–shape changes, rather than direct action of the ionic media.

Similar results, due to the volume–shape changes produced, are found with variation in the cell pH. An optimum rate of uptake occurs at pH 7·4.

Metabolic considerations. The initial rate of uptake of oxygen by reduced sheep erythrocytes, measured within 3 hr of collecting the blood sample, was found to be considerably higher at room temperature than the values observed in the preceding experiments, which were outside this limit. No similar effect was observed with carbon monoxide, unless oxygen was also present. A decay curve of the initial rate with time, obtained on human erythrocytes, indicates that the cells reach a passive level within 2 hr, at room temperature and in the absence of oxygen. The process is inhibited by NaF and no advantage was obtained on suspending the cells in their own plasma, rather than Ringer–Locke solution. An analysis of the results suggests that the membrane permeability is increased by cell metabolism.

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Effect of physical training on the physical fitness of women students.

By A. W. SLOAN. *Department of Physiology and Pharmacology, University of Cape Town*

Regular physical training has been found to increase physical fitness as estimated by exercise tolerance tests. One such test, the Harvard step test (Brouha, Graybiel & Heath, 1943), gives a useful indication of the capacity of healthy subjects for strenuous physical work. Fitness is expressed as an index (F.I.), which depends directly on the period of exercise and inversely on the pulse rate thereafter. Originally designed for men the test is modified for women by lowering the height of the step (Hardy, Clarke & Brouha, 1943).

To ascertain the effect of different amounts of physical training on the ability of young women to perform this test, four groups of student-teachers at Cape Town Training College were tested at the beginning of the school year and again 4 and 9 months later. One group, students of physical education, had a strenuous programme of gymnastics, dancing and games. Students of infant-school teaching and some students attending a general course had much less physical training; a fourth group of students had none at all.

As well as the F.I. estimation the resting pulse (R.P.) of each student was counted, usually on a different day from the step test. Each student's height and weight was measured and she was asked whether she was menstruating at the time of the test.

At the beginning of the investigation the students who were about to specialize in physical education had already a higher F.I. than any of the other groups. After approximately 4 months of the various courses the physical education students had a significantly higher F.I. than at the first set of tests; this improvement was maintained without further significant change over the next 5 months. No other significant changes in F.I. occurred but, by the end of the first 4 months, there was a significant difference in F.I. between the general course students who did gymnastics and those who did not; this difference was maintained at the later tests.

As in a previous investigation on men (Keen & Sloan, 1958) there was no significant correlation in the group as a whole between F.I. and either height or weight. There was no consistent correlation between F.I. and R.P. and there was no evidence that menstruation had any effect on the F.I.

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Observations on the formation of kinins in human plasma collected during labour. By DESIRÉE ARMSTRONG, C. A. KEELE and J. W. STEWART. *The Middlesex Hospital Medical School, London, W.* 1

The formation of kinins in the plasma of women, shortly before, during and after labour, was studied by using the following procedures, or materials, for activation: exposure to glass (Armstrong, Jepson, Keele & Stewart, 1957); dilution (Schachter, 1956); human urine (cf. Gaddum & Horton, 1959); human plasmin (Armstrong *et al.* 1957; Lewis, 1958); disodium edetate or ε-amino caproic acid (Armstrong, Keele & Stewart, unpublished observations).

'Cell-free, pre-active' heparinized plasma (Armstrong *et al.* 1957) was prepared from blood collected from the antecubital vein, and stored at −20° C until required for testing (usually 1–10 days).

Quantitative measurements of kinin production were made by the isolated rat uterus technique. The activity in samples of the mother's plasma collected in the immediate antenatal and post-natal period was compared with that of samples collected during labour, and with a normal control.

Our results indicate that kinin production in plasma from pregnant women is normal until the commencement of labour, when it begins to decrease, reaching its lowest values around the end of the second stage; thereafter, the ability to form kinins returns. In some cases normal values were obtained at 4 hr post partum, whilst, in others, somewhat low values persisted in samples collected at 1 or 2 days post partum.

Out of 20 mothers whose plasma was collected at the end of the second stage of labour 17 gave low values for kinin production. Quantitative measurements indicated that in at least ten of these kinin production was below 10 % of the normal and in four it was well below 5 %.

No evidence for the presence of an inhibitor of the rat uterine response, of increased kinin destruction, or for the presence of a blocking agent for the kininogen has been found in the plasmas. 'Contact factor' (Margolis, 1958) production was not deficient. The poor kinin formation appears to be due to a deficiency of the substrate from which the kinins arise, and not to any defect of the activation system.

We have considered, and excluded, the effects of starvation, and of drugs used in the course of labour (e.g. pethidine, nitrous oxide, ergometrine) as possible causes of the substrate defects. We have not induced the defect by severe exercise, by adrenaline infusion or by corticotrophin administration in non-pregnant women.

The physiological significance of the findings is not known.

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The formation of plasma kinin by the action of human plasmin on human plasma. By V. Eisen and C. A. Keele. *Department of Pharmacology, Middlesex Hospital Medical School, London, W.* 1

When applied to an exposed blister base in human skin, a preparation of *human plasmin* (Thrombolysin, Merck) in a concentration of 0·1–1 mg/ml. in physiological saline produced pain. A single application desensitized the area to subsequent applications. Administration of 1 mg of plasmin to a 5 ml. bath caused delayed (100–120 sec) rhythmic contractions of the isolated rat uterus.

The addition of human plasmin to oxalated or heparinized *human plasma* which had not been in contact with glass ('intact' or 'pre-active' plasma) caused the rapid appearance of large amounts of plasma kinin, detected by the production of pain and by contraction of the rat uterus (latent period 5–10 sec). When the final concentration of plasmin in plasma was 1 mg/ml., plasma kinin was detectable in 0·1 ml. of the mixture within 15–30 sec; its concentration was quite high at 2 min and reached a peak at about 5 min; the concentration then fell to low values at 20–30 min. This plasma kinin production was inhibited by the soya bean trypsin inhibitor (2 mg/ml.).

When plasmin was added to plasma which had previously been shaken with glass ballotini to remove component B (Margolis, 1958) the developed kinin concentration was only $\frac{1}{5}$–$\frac{1}{10}$ of that produced in intact plasma. Conversely, prior treatment of plasma with plasmin greatly reduced kinin formation by subsequent contact with glass. In concentrations of 0·1–1 mg/ml., plasmin forms plasma kinin mainly by reacting with component B which is also essential for activation by glass. The reaction with component B might be direct or through activation of component A (Margolis, 1958).

The action of human plasmin on intact human plasma is as rapid as that of glass (Armstrong, Jepson, Keele & Stewart, 1957) and we would therefore describe plasmin as a quick-acting plasma-kinin-forming enzyme (cf. Lewis, 1958, 1959).

The threshold concentration of plasmin for the formation of plasma kinin in intact plasma is about 0·05–0·1 mg/ml. It is presumed that lower

concentrations cannot overcome the inhibitory effects of the anti-plasmin which occurs in plasma.

One of us (V. E.) is working on a grant from the Empire Rheumatism Council. We are grateful to Dr J. J. F. Merry of Merck, Sharp and Dohme Limited for helping us obtain samples of Thrombolysin.

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The cutaneous vasomotor control in the human nose, lip and chin. By R. H. Fox, R. Goldsmith and D. J. Kidd. *Division of Human Physiology, National Institute for Medical Research, London, N.W.* 3

It has previously been shown that the vasodilatation in the ear accompanying body heating is produced by inhibition of vasoconstrictor tone, whereas in the forehead it is mainly the result of an active vasodilator mechanism (Fox, Goldsmith & Kidd, 1960). In the present experiments, with similar techniques, the study has been extended to investigate the mechanisms in the nose, glabrous portion of the lip, and the chin. In addition, experiments have been performed to see whether gustatory sweating of the face is attended by a cutaneous vasodilatation.

The subjects were three fit, male adults. Forearm blood flow was measured by venous-occlusion plethysmography, and two surface calorimeters were used to detect changes in the rate of blood flow through the skin of the face. The calorimeters were placed symmetrically on the two sides of the face, and the effects of blocking the cutaneous nerve supply to one side of the face on the vasodilatation and vasoconstriction accompanying body heating and cooling were then observed. The gustatory response was investigated in experiments in which a calorimeter was placed on one side of the forehead, cheek near the nose, or chin, with a sweat-collecting capsule placed symmetrically on the other side. The effects on sweat production and skin blood flow of chewing chillies were observed.

The results show that release of vasoconstrictor tone is the dominant mechanism in the vasodilatation of the glabrous portion of the lip. The release of vasoconstrictor tone also plays an important part in the vasomotor control of the skin of the nose.

The skin of the chin, distant from the lower lip, is like that of the forehead and forearm in having a dominant active vasodilator mechanism

and only a small component of control by release of vasoconstrictor tone. Gustatory sweating of the chin, cheek and forehead elicited by chewing chillies is accompanied by a cutaneous vasodilatation, a finding which is in agreement with the prediction of Fox & Hilton (1958).

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The effect of changes in blood flow on the calibre of large arteries.

By J. L. D'Silva and R. F. Fouché. *Departments of Radiology and Physiology, London Hospital, London, E.* 1

It is not uncommon in clinical practice to find abnormally wide arteries supplying lesions associated with an increased blood flow. This communication concerns the effect of changes in blood flow on the calibre of the femoral artery and its large branches. The observations were made on cats anaesthetized with Nembutal (pentobarbitone; Abbott Laboratories); the calibre of the arteries was shown by angioradiography. Changes in flow were produced in one limb, the other acting as a control, and variations in flow were produced by two different methods:

(1) Repeated contractions of the calf muscles were used to produce hyperaemia. Direct muscle stimulation was found to be preferable to sciatic nerve stimulation as the latter produced narrowing of arteries in the contralateral leg unless the nerve was cut and the stimulus applied to the peripheral end. The parameters used increased the flow from the femoral vein two- or threefold.

(2) A connexion was made between the anterior tibial artery of one leg and a vein of the other leg, a tap in the circuit permitting the arterio-venous (A-V) fistula to be opened or closed. Precise measurements of flow were not made but flow was probably increased several fold with the tap open. Neither method was associated with any significant change in carotid artery blood pressure.

The results of both types of experiment demonstrated widening of large arteries with increased flow and narrowing with decreased flow. During exercise hyperaemia the femoral artery widened by about 30%, whereas small muscular branches more than doubled in calibre and many arterial channels, previously invisible, became visible.

With the A–V fistula open the femoral artery widened by about 30%. The increase in calibre was confined to the femoral and popliteal arteries and the segment of the anterior tibial artery proximal to the fistula. None

of the other branches of the femoral artery appeared to be affected. The changes in calibre occurred within a few seconds of the change of flow.

The changes in calibre of arteries during exercise hyperaemia confirm previous observations (Hilton, 1956, and others). These workers suggested that the calibre change was produced by muscle metabolites initiating an axon reflex, but it seems unlikely that this is the explanation of the vasodilatation associated with an open A–V fistula. In this case, the change in flow itself may have been the stimulus for change in calibre. If this is so, the change in calibre associated with exercise hyperaemia may have been produced similarly.

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